IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Padigaru et al.		
SERIAL NUMBER:	09/800,321	EXAMINER:	Misook Yu
FILING DATE:	March 5, 2001	ART UNIT:	1642
FOR:	NOVEL PROTEINS AND NUCLEIC ACIDS ENCODING SAME		

DECLARATION OF JOHN ROBERT MACDOUGALL UNDER 37 C.F.R. § 1.132

I, John Robert MacDougall, declare and state that:

1. I received a Bachelor of Science degree in toxicology from the University of Guelph, Guelph, Ontario, Canada in 1990, and a Ph. D. degree in Medical Biophysics from the University of Toronto, Toronto, Ontario, Canada in 1995 (CV attached hereto);

2. I am currently employed at CuraGen Corporation in New Haven, Connecticut, as a Senior Research Scientist, Preclinical Development/Oncology, the assignee of this patent application;

3. I am the first author of eight (8) scientific papers which appear in Journals such as the Cancer and Metastasis Reviews, Cellular Immunology, Molecular and Cellular Differentiation, and a book chapter in Breast Cancer (Humana Press);

4. I have read and am familiar with the specification and claims of United States Patent Application USSN. 09/800,321 entitled "Novel Proteins and Nucleic Acids Encoding Same", including the March 22, 2004 Office Action;

5. I have performed, or have had performed under my supervision, scientific studies evaluating the expression of the NOV1b protein (CG55343) in isolated normal and pathological human tissues by immunohistological techniques. Breifly, formalin fixed

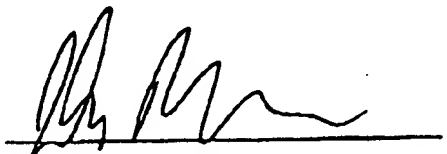
and paraffin embedded tissue microarrays, purchased from Cybrdi Inc (Gaithersburg MD) typically comprising approximately 60 tissue specimens were cut in 5 □m sections and were rehydrated through washes in xylene and a graded series of ethanols terminating in PBS. Endogenous peroxidase activity was quenched in a 3% solution of hydrogen peroxide (Fisher Scientific, Hampton, NH) in methanol. Staining was then carried out under standard conditions. Briefly, tissue sections were blocked in a solution of 5% BSA (Sigma, St. Louis MO) and 1% goat serum (Jackson Immunoresearch laboratories, West Grove, PA) in PBS for 1 hour. Subsequently, the sections were incubated with affinity purified rabbit anti-CG55343 polyclonal antibody or matched non-specific control (rabbit IgG; Jackson Immunoresearch) diluted in blocking buffer. After 1 hour at room temperature the sections were washed in 3 changes of PBS for 5 to 10 minutes each. The sections were then incubated with Dako Envision anti-rabbit HRP conjugated polymer reagent (DakoCytomation, Carpenteria, CA) for 30 minutes according to the manufacturer's instructions. Sections were washed and then incubated for 5 minutes with DAB substrate reagent (DakoCytomation). The sections were rinsed in deionized water and were counterstained in hematoxylin (Fisher Scientific), dehydrated through alcohol and xylene and coverslipped with permount (Fisher Scientific). Staining was evaluated under a microscope at 10-40X magnification for the presence of brown precipitate where primary antibody bound to CG55343 in the tissue.

6. Breast specimens studied included fifty three infiltrating ductal carcinomas; three samples of normal mammary gland; six fibrous fatty tissue and one sample of medullary carcinoma. All samples of infiltrating ductal carcinomas stained positive, indicating the presence of CG55343. In most samples, staining was uniform across cells, however a few specimens showed heterogeneous staining. Fibrous fatty tissue and medullary carcinoma specimens were negative. Samples of normal mammary gland were positive only in the ductal epithelial cells. See Figure 1. The majority of lung adenocarcinoma (23 of 26), squamous cell (17 of 18) and bronchioalveolar carcinoma (5 of 5) specimens were positively stained indicating the presence of CG55343. Staining was also detected in

papillary alveolar carcinoma and in normal high columnar epithelium and brush border cells. Small cell (7 of 8) and large cell (2 of 3) lung cancers were weakly positive.

7. While CG55343 was detected in normal mammary ductal epithelial cells, it is understood that malignancy is the uncontrolled proliferation of what otherwise might be normal cells. Particularly in the breast ductal carcinomas and lung adenocarcinoma specimens studied, the proliferation of epithelial derived cells resulting in the cancer also results in an abundance of strongly stained cells, indicating abundant expression of CG55343 protein in these specimens as compared to normal tissues. As demonstrated, the increased expression of CG55343 is correlated with the presence of these cancers. It is therefore my belief and scientific opinion that increased expression of CG55343 correlates with the presence of breast and lung carcinoma as demonstrated in these samples isolated from patients.

8. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that willful false statements may jeopardize the validity of this application and any patent issuing therefrom.



John Robert MacDougall

Signed this day 21 of September, 2004

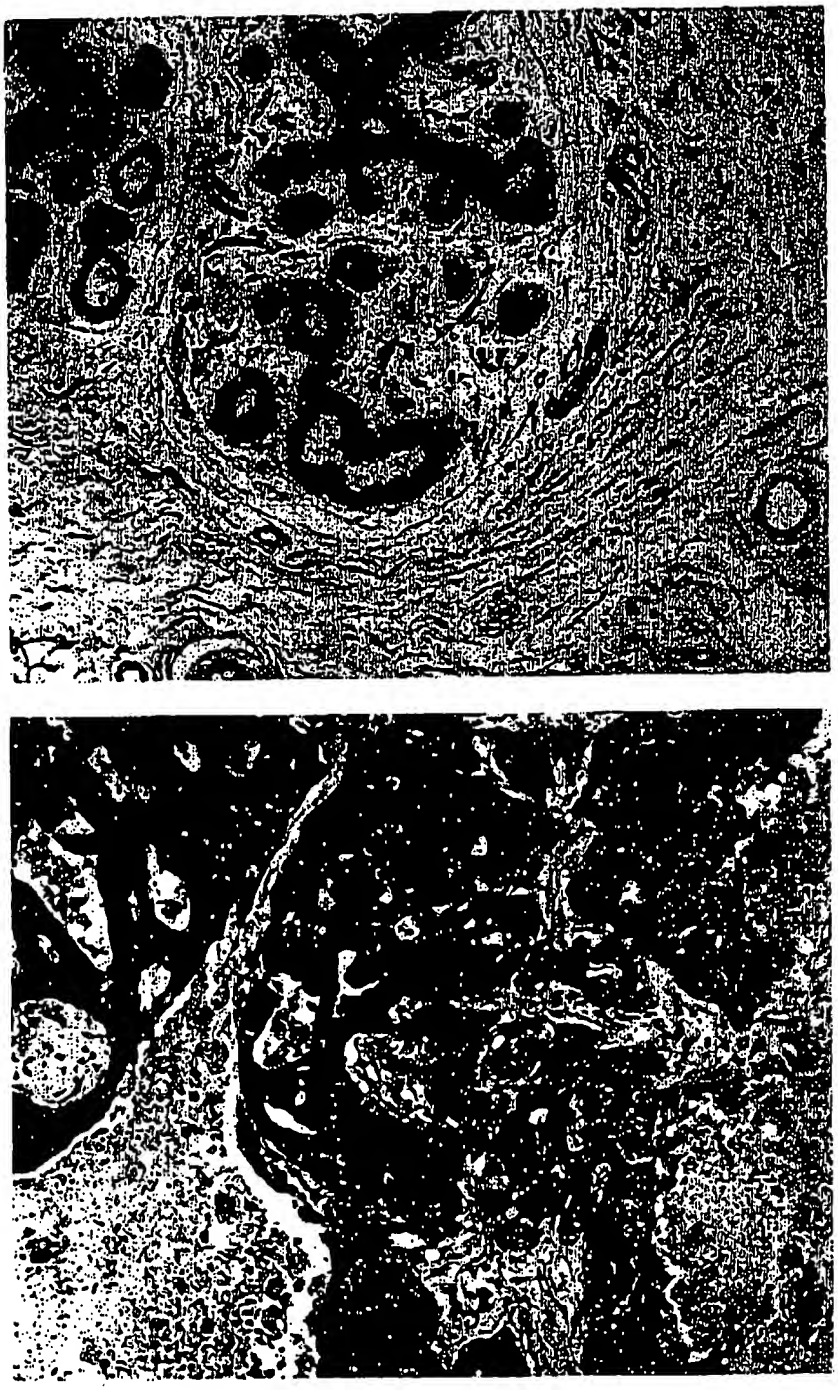


Figure 1. CG55343 in normal mammary ductal epithelium (top) and breast adenocarcinoma (bottom) detected using anti-CG55343 antibody.

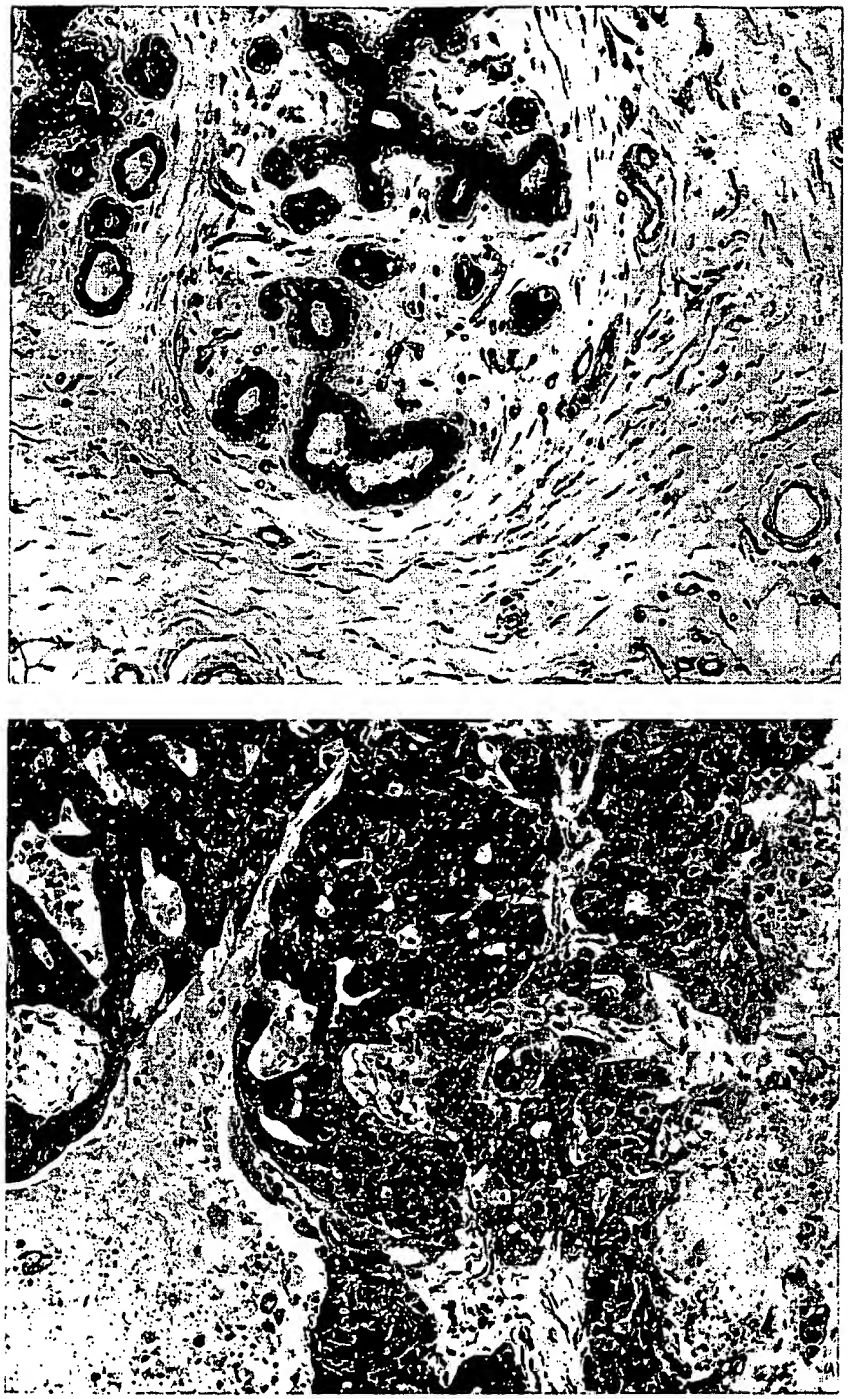


Figure 1. CG55343 in normal mammary ductal epithelium (top) and breast adenocarcinoma (bottom) detected using anti-CG55343 antibody.

Curriculum Vitae

Personal information

Name:	John Robert MacDougall
Date of birth:	February 4th 1967
Place of birth:	St. Catharines, Ontario, Canada
Citizenship:	Canadian; Currently authorized on TN visa, Green Card application underway

Education

1986-1990	Specialized Honours Bachelor of Science Degree in Toxicology obtained with Distinction from the University of Guelph, Guelph, Ontario, Canada.
1990-1995	Doctoral Program, Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada. Supervisor, Dr. R.S. Kerbel Thesis title: "Phenotypic switches in the malignant progression of human melanoma"

Research Experience

1995-1998	Post-Doctoral Fellow under supervision of Dr. Lynn Matrisian, Department of Cell Biology, Vanderbilt University, Nashville, Tennessee, U.S.A.
1998-2000	Career Scientist, Cancer Care Ontario. Northwestern Ontario Regional Cancer Centre, Thunder Bay, Ontario, Canada.
2000-Present	Senior Research Scientist, Preclinical Development/Oncology, CuraGen Corporation. Branford, Connecticut, U.S.A.

Appointments

1999-2000	Adjunct Professor, Department of Biology, Lakehead University, Thunder Bay, Ontario, Canada.

Awards, Scholarships and Honours

1989	Natural Sciences and Engineering Research Council of Canada Studentship Award.
1990-1995	Medical Research Council of Canada Studentship Award
1995	National Cancer Institute of Canada Fellowship Award (declined)
1995-1998	Susan G. Komen Breast Cancer Foundation Fellowship Award

Funding History

1998	Cancer Care Ontario/Northern Cancer Research Foundation start-up funds ($80,000)
1998	Andison Foundation: Characterization of a novel S100 protein in the progression of breast cancer ($20,000/yr for 2 years).
1999	Northern Cancer Research Foundation: Identification and characterization of a repressor of gene expression in breast cancer progression ($60,000/yr for 2 years)
1999	Canadian Breast Cancer Research Initiative: Repression of Estrogen Receptor Expression as a mechanism driving breast cancer tumor progression ($50,000 over 2 years)

Committees

1998	Susan G. Komen Foundation Fellowship Review Panel

1999-2000	Research Advisory Committee, Cancer Care Ontario
1999-2000	Thunder Bay Regional Hospital Research Institute Task Force
2001-2002	Chair, CuraGen Internal Protein Therapeutic Project Committee
2001	US Armed Forces (UASMRMC) Breast Cancer Review Panel: Pathobiology #1
2002	US Armed Forces (UASMRMC) Breast Cancer Review Panel: Pathobiology #3

Memberships in Professional Societies

1998-Present American Association for Cancer Research (Member #10733)

Teaching

1998	Special lecture in Angiogenesis in Cancer, Department of Pathology, Vanderbilt University
2000	Cancer Biology 4230, Department of Biology, Lakehead University

Journal Reviewer

American Journal of Pathology
Cancer Research
Journal of Biological Chemistry
Molecular and Cellular Biology
Clinical Cancer Research
British Journal of Cancer
International Journal of Cancer
Molecular Carcinogenesis
Molecular Biology of the Cell

Invited presentations

1994	Regulation of the 92-kDa gelatinase B during tumor progression in human melanoma. Grand Oncology Rounds, Toronto-Bayview Regional Cancer Centre, Toronto, Ontario.
1997	Repression of gene expression in malignancy and differentiation of mammary epithelial cells. Division of Cancer Biology Research, Sunnybrook Health Science Centre, Toronto, Ontario.
1998	Targets of repression in tumor progression and cellular differentiation. Northwestern Ontario Regional Cancer Centre, Thunder Bay, Ontario.
1998	Repression of gene expression in tumor progression and cellular differentiation. Ottawa Regional Cancer Centre, Ottawa, Ontario.
1999	What makes a scientist? Lakehead Rotary International.
1999	Where does basic science fit in cancer diagnosis, treatment and care? Thunder Bay District Breast Cancer survivors support group.
1999	Repression of gene expression in tumor progression. Department of Biology, Lakehead University.
1999	Environmental Exposure and Cancer Causation. Women's Decade Council Conference.
1999	Targets of gene extinction in tumor progression and cellular differentiation. Eli Lilly and Co. Research Labs and Corporate Headquarters, Indianapolis, Indiana.
2000	Targets of gene extinction in tumor progression and cellular differentiation. CuraGen Corporation, Branford Connecticut, U.S.A.
2002	First Annual Clinical Genomics Symposium: Northeast Region. Sponsored by Ardais Corporation
2002	First Annual Clinical Genomics Symposium: Mid-Atlantic Region. Sponsored by Ardais

Corporation

Publications

i) *Refereed papers, Published or in press*

1) **MacDougall, J.R.**, Croy, B.A., Chapeau, C. and D.A. Clark. Demonstration of a splenic cytotoxic effector cell in mice of genotype SCID/SCID.BG/BG. **Cellular Immunology** 130:106-117 (1990).

2) **MacDougall, J.R.**, Kobayashi, H. and R.S. Kerbel. Responsiveness of normal, dysplastic melanocytes and melanoma cells from different lesional stages of progression to the growth inhibitory effects of TGF-β. **Molecular and Cellular Differentiation**, 1(1):21-40 (1993).

3) Graham, C.H., Hawley, T.S., Hawley, R.G., **MacDougall, J.R.**, Kerbel, R.S., Khoo, N. and P.K. Lala. Establishment and characterization of first trimester human trophoblast cells with extend lifespan. **Experimental Cell Research**, 206:204-211 (1993).

4) Kobayashi, H., Man, S., **MacDougall, J.R.**, Graham, C.H. Lu, C. and R.S. Kerbel. Variant sublines of early-stage human melanomas selected for tumorigenicity express a multicytokine resistant phenotype. **American Journal of Pathology**, 144(4):776-786 (1994).

5) Graham, C.H., Connelly, I., **MacDougall, J.R.**, Kerbel, R.S., Stetler-Stevenson, W.G. and P.K. Lala. Resistance of malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor-β. **Experimental Cell Research** 214:93-99 (1994).

6) **MacDougall, J.R.** and R.S. Kerbel. Constitutive production of 92-kDa gelatinase B is suppressed by alterations in cell shape. **Experimental Cell Research** 218:508-515 (1995).

7) **MacDougall, J.R.**, Bani, M.R., Lin, Y., Rak, J.W. and R.S. Kerbel. The 92-kDa gelatinase B is expressed by advanced stage melanoma cells: Suppression by somatic cell hybridization with early stage melanoma cells. **Cancer Research** 55:4174-4181 (1995).

8) **MacDougall, J.R.**, Bani, M.R. Lin, Y., Muschel, R. and R.S. Kerbel. "Proteolytic Switching": Opposite patterns of regulation of gelatinase B and its inhibitor TIMP-1 during human melanoma progression and consequences of gelatinase B overexpression. **British Journal of Cancer** 80:504-512 (1999).

9) **MacDougall, J.R.** and L.M. Matrisian. Targets of extinction: Identification of genes whose expression is repressed as a consequence of somatic fusion between cells representing basal and luminal mammary epithelial phenotypes. **Journal of Cell Science**, 113(3): 409-423 (2000).

10) Haro, H., Crawford, H., Fingleton, B.F., **MacDougall, J.R.**, Shinomiya, K., Spengler, D.M. and L.M. Matrisian. Matrix metalloproteinase-3-dependent generation of a macrophage chemoattractant in a model of herniated disc resorption. **Journal of Clinical Investigation**, 105(2): 133-141 (2000).

11) Sadatmansoori,S. **MacDougall,** J. Khademi, S. Cooke, L.S., Guarino, L., Meyer, E.F. and Reza Forough. Construction, Expression, and Characterization of a Baculovirally Expressed Catalytic Domain of Human Matrix Metalloproteinase-9
Sepideh Sadatmansoori, John MacDougall, Shahram Khademi, Laurence S. Cooke, Linda Guarino, Edgar F. Meyer, Reza Forough **Protein Expression and Purification** 23(3) 447-452 (2001).

ii) *Review papers*

1) **MacDougall, J.R.** and L.M. Matrisian. Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. **Cancer and Metastasis Reviews** 14:351-362 (1995).

2) **MacDougall, J.R.** and L.M. Matrisian. Matrix metalloproteinase expression in breast cancer progression. In A. Bowcock (ed.): **Breast Cancer**, Humana Press (1998).

iii) *Non-refereed papers, published or in press*

1) Kerbel, R.S., Cornil, I. and **J.R. MacDougall**. Transition in responsiveness to multiple inhibitory growth factors: Possible contribution to tumor progression and metastasis. In, Rabes, H., Peters, P.E. and K. Munk (eds.): Metastasis: Basic research and its clinical applications. Contributions to Oncology. Karger, Basel. 44:185-202 (1992).

2) Kerbel, R.S. and **J.R. MacDougall**. Possible contribution of growth factors to the evolution of metastases and *de novo* multidrug resistance in cancer. In B.A. Teicher (ed.): Drug resistance in Oncology, Marcel Drekker, New York. pp 583-602 (1993).

3) Lu, C., **MacDougall, J.R.**, Kobayashi, H. and R.S. Kerbel. Transition states in multicytokine responsiveness during human malignant melanoma progression: Implications for metastasis. In Marks, P.A., Turler, H. and R. Weil (eds.): Precancerous lesions: A multidisciplinary approach. Challenges of Modern Medicine. Ares-Serono Symposia Publications. pp 167-175 (1993).

v) *Abstracts and presentations, Published or in press*

1) **MacDougall, J.R.**, Croy, B.A., Chapeau, C. and D.A. Clark. Assessment of cellular Immune responses in *scid/scid.bg/bg* mice. **Proceedings of the Canadian Society of Immunology.** (1990).

2) **MacDougall, J.R.**, Kerbel, R.S. and B.A. Croy. Differences in the growth of the human melanoma cell line "MeWo" in SCID and SCID-beige mice as compared to nude mice. **Proceedings of the Canadian Society of Immunology.** (1990).

3) **MacDougall, J.R.** and R.S. Kerbel. Expression of 92 kDa gelatinolytic activity correlates with malignant progression of melanoma cells and is stimulated by TGF-β. **Clinical and Experimental Metastasis** 10 (supplement 1):82 (1992).

4) **MacDougall, J.R.**, Lin Y., and R.S. Kerbel. 92 kDa gelatinase B production by advanced stage melanoma cells is lost following somatic hybridization with early stage melanoma cells. Selected for presentation at the Biology 23 minisymposim slide presentation, Annual meeting of the American Association for Cancer Research, San Francisco, CA. April 10-13 1994. **Proceedings of the American Association for Cancer Research** (1994).

5) **MacDougall, J.R.**, Rudolph, L.A., Arteaga, C.A. and L.M. Matrisian. The expression of the matrix metalloproteinase matrilysin is controlled by both positive and negative factors in breast cancer progression. **Proceedings of the American Association for Cancer Research** (1997).

6) **MacDougall, J.R.** and L.M. Matrisian. Targets of repression: identification of genes whose expression is repressed as a consequence of somatic cell fusion. **Proceedings of the American Association for Cancer Research** (1998).

7) Kubinec, M and **J.R. MacDougall**. Mutual exclusivity of patterns of gene expression in somatic hybrid cells: Heterokaryon analysis reveals a role for a soluble, cytoplasmic factor determining the luminal and basal mammary epithelial phenotypes. **Proceedings of the American Association for Cancer Research** (2000).

8) Shi, Y., Richard, C., Yau, J., Vergidis, R., Kubinec, M., Th'ng, J. and **J.R. MacDougall**. Expression of "clone-10" in human primary breast cancer, a potential marker for disease progression. **Proceedings of the American Association for Cancer Research** (2002).

9) Rastelli, L., Ju, J., Malyankar, U., Simons, J., Huang, C., Shimkets, J., Taillon, B., Herrmann, J. and **J.R. MacDougall**. The melanoma vascular mimicry phenotype defined in large-scale gene expression analysis. **Proceedings of the American Association for Cancer Research** (2002).

Meetings in attendance

Eighty-first Annual Meeting of the American Association for Cancer Research, Washington D.C. May 23-26 1990.

Gordon Conference on Cancer, Newport, Rhode Island, August 12-16, 1991.

The thirty-fifth Annual Clinical Conference and Twenty-fourth Annual Special Pathology Program: Advances in the Biology and Clinical Management of Melanoma, Houston, Texas, November 20-23 1991.

Fourth International Congress of the Metastasis Research Society: Science and Medicine in Cancer Metastasis, Paris, France, August 31-September 4 1992.

Gordon Conference on Matrix Metalloproteinases, Plymouth, New Hampshire, August 15-20, 1993.

Eighty-fifth Annual Meeting of the American Association for Cancer Research, San Francisco CA. April 10-13 1994.

AACR special conference: Proteases and Protease Inhibitors. Panama City Beach, Florida. March 1-5, 1996.

Sixth International Congress of the Metastasis Research Society. Gent, Belgium September 8-11 1996.

Eighty-eighth Annual Meeting of the American Association for Cancer Research, San Diego CA. April 11-14, 1997.

Eighty-ninth Annual Meeting of the American Association for Cancer Research, New Orleans LA. March 28-April 1, 1998.

Ninetieth Annual Meeting of the American Association for Cancer Research, Philadelphia PA. April 10-14, 1999.

Ninety-first Annual Meeting of the American Association for Cancer Research, San Francisco, CA. April 1-5, 2000.

Ninety-third Annual Meeting of the American Association for Cancer Research, San Francisco, CA. April 6-10, 2002.

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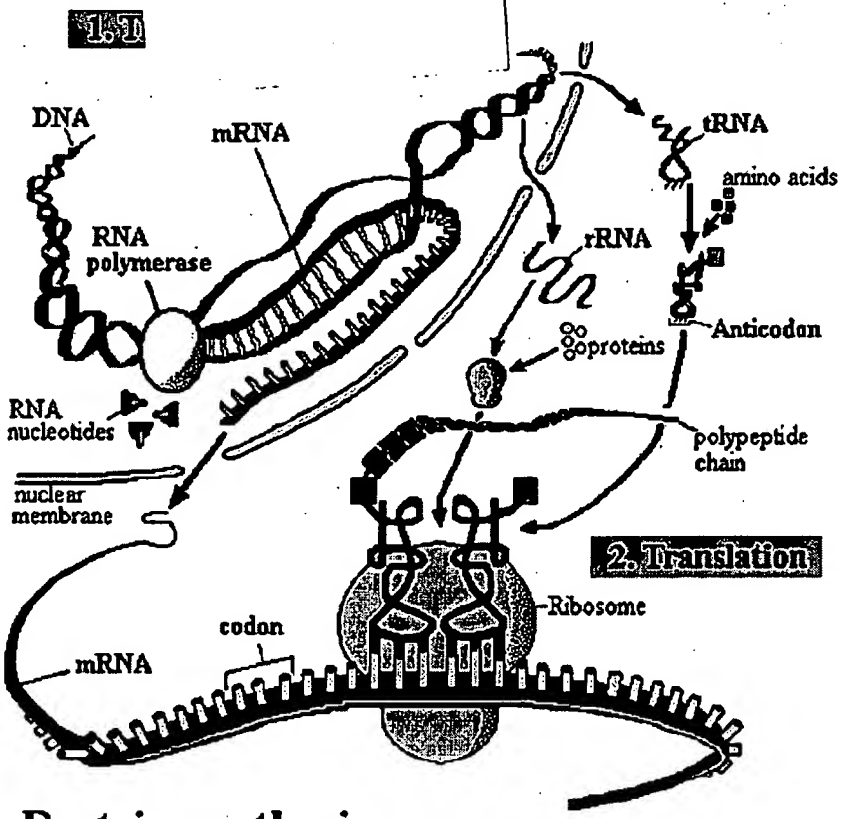
ABOUT BIOTECH

Graphics Gallery

Appendix A

thesis



Protein synthesis

Legend:
Process whereby DNA encodes for the production of amino acids and proteins.

This process can be divided into two parts:

1. Transcription
Before the synthesis of a protein begins, the corresponding RNA molecule is produced by RNA transcription. One strand of the DNA double helix is used as a template by the RNA polymerase to synthesize a messenger RNA (mRNA). This mRNA migrates from the nucleus to the cytoplasm. During this step, mRNA goes through different types of maturation including one called **splicing** when the non-coding sequences are eliminated. The coding mRNA sequence can be described as a unit of three nucleotides called a **codon.**

2. Translation
The ribosome binds to the mRNA at the **start codon** (AUG) that is recognized only by the initiator tRNA. The ribosome proceeds to the elongation phase of protein synthesis. During this stage, complexes,

composed of an amino acid linked to tRNA, sequentially bind to the appropriate codon in mRNA by forming complementary base pairs with the tRNA **anticodon**. The ribosome moves from codon to codon along the mRNA. Amino acids are added one by one, translated into polypeptidic sequences dictated by DNA and represented by mRNA. At the end, a release factor binds to the stop codon, terminating translation and releasing the complete polypeptide from the ribosome.

One specific amino acid can correspond to more than one codon. The genetic code is said to be degenerate.

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Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10578–10582, November 1992
Genetics

Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease

(mammary tumorigenesis/protein-tyrosine kinase/cancer metastasis)

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*Institute for Molecular Biology and Biotechnology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada, L8S 4K1; ‡Department of Pathology, School of Medicine, University of California, Davis, CA 95616; and †Department of Microbiology and Cancer Center, University of Virginia School of Medicine, Charlottesville, VA 22908

Communicated by Philip Leder, July 30, 1992

ABSTRACT **Overexpression and amplification of the *neu* (c-*erbB2*, *ERBB2*) protooncogene have been implicated in the development of aggressive human breast cancer. To directly assess the effect of mammary gland-specific expression of the *neu* protooncogene, transgenic mice carrying unactivated *neu* under the transcriptional control of the mouse mammary tumor virus promoter/enhancer were established. By contrast to the rapid tumor progression observed in several transgenic strains carrying the activated *neu* transgene, expression of unactivated *neu* in the mammary epithelium resulted in the development of focal mammary tumors after long latency. The majority of the mammary tumors analyzed expressed elevated levels of *neu*-encoded mRNA and protein. Overexpression of *neu* in the mammary tumors was also associated with elevated neu intrinsic tyrosine kinase activity and the *de novo* tyrosine phosphorylation of several cellular proteins. Interestingly, many of the tumor-bearing transgenic mice developed secondary metastatic tumors in the lung. These observations suggest that overexpression of the unactivated neu protein can induce metastatic disease after long latency.**

The *neu* protooncogene encodes a 185-kDa transmembrane protein that is a member of the epidermal growth factor receptor family (1–4). Oncogenic activation of *neu* can occur through a point mutation in the transmembrane domain (5), deletion of the extracellular domain (6), or overexpression (7–9). Amplification and overexpression of the human homologue of *neu* (c-*erbB2*; human gene symbol, *ERBB2*) have been observed in a large percentage of primary breast cancers (1, 10–13) and appear to be inversely correlated with the survival of the patient (11, 13).

Given the close correlation between *neu* overexpression and mammary carcinogenesis, a number of laboratories have been interested in directly testing the tumorigenic potential of the *neu* oncogene in the mammary epithelium of transgenic mice. Initially, this was accomplished by generating several lines of transgenic mice carrying the activated rat *neu* oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer (14–16). In several strains of MMTV/activated *neu* mice, early onset of transgene expression in the mammary epithelium of female mice was associated with the synchronous development of tumors involving the entire mammary epithelium. These results suggested that expression of activated *neu* requires few, if any, additional genetic events to transform the mammary epithelial cell (14).

These studies suggested that activated *neu* can act as a potent oncogene in the mammary epithelium. However, overexpression of the unactivated neu protein may be the primary mechanism contributing to human breast cancer, since examination of primary breast cancer biopsy samples has thus far failed to reveal comparable activating mutations (13, 17). To directly test the oncogenic potential of the unactivated neu protein in the mammary epithelium, six lines of transgenic mice carrying a MMTV/unactivated *neu* fusion gene were derived. Overexpression of the unactivated *neu* product in the female mammary epithelium of five of these lines resulted in appearance of focal mammary tumors that metastasized with high frequency. These observations support the hypothesis that elevated expression of *neu*-associated tyrosine kinase activity in the mammary epithelium induces metastatic disease.

MATERIALS AND METHODS

DNA Constructions and Generation of Transgenic Mice. The recombinant plasmid pMMTVneuN was established by inserting the *Hin*dIII–*Eco*RI fragment encoding the unactivated *neu* cDNA and simian virus 40 (SV40) polyadenylylation and splicing signals from pSV2neuN (5) into the corresponding *Hin*dIII–*Eco*RI sites of the plasmid pMMTVneuNT (14). The plasmid pASV, which contains SV40 early splice and polyadenylylation signals, was constructed as described (14). The β-casein riboprotection probe was obtained from J. Rosen (Baylor College of Medicine) and was cloned as a 205-base-pair *Pst* I fragment in the plasmid pSP64 (Promega). The internal control plasmid rpL3227.3.7 was obtained from M. Shen (Harvard Medical School) and encodes the *Xho* II–*Dra* I fragment of the mouse ribosomal protein gene *rpL32* inserted into the corresponding sites of the plasmid pBluescript KS (Stratagene). All plasmid DNAs were isolated and purified as described by Sinn *et al.* (18). To derive transgenic mice carrying the MMTV/unactivated *neu* transcription unit, the pMMTVneuN *Sph* I–*Eco*RI fragment was microinjected into the pronuclei of FVB/N fertilized one-cell zygotes as described (18). Transgenic progeny were identified by probing genomic DNA after Southern blot transfer (19) with a *neu* cDNA-specific probe that was radiolabeled with [α-^{32}P]dCTP by random priming (20).

Expression Analyses. RNA was isolated from tissues by the procedure of Chirgwin *et al.* (21), using a CsCl sedimentation gradient modification. RNA probes were made with either pBluescript (Stratagene, San Diego, CA) or pGEM (Promega) vectors, and RNase protection was done according to Melton *et al.* (22), with 10 μg of total cellular RNA per assay. Western analyses with rabbit anti-phosphotyrosine antibodies were performed as described (23). To detect the presence of the neu protein in these tumors, 300 μg of total cellular protein was

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Abbreviations: MMTV, mouse mammary tumor virus; SV40, simian virus 40.

§To whom reprint requests should be addressed.

electrophoresed in an SDS/8% polyacrylamide gel, transferred to nitrocellulose, and probed with the anti-neu monoclonal antibody 7.16.4 (24). The proteins were visualized with the enhanced chemiluminescence (ECL) detection system (Amersham). *In vitro* kinase assays were conducted as described (16).

Histological Evaluation. Complete autopsies were performed and both gross and microscopic examinations were done. Tissues were fixed in 4% paraformaldehyde, blocked in paraffin, sectioned at 4 μm, routinely stained with hematoxylin and eosin, and examined as indicated in Fig. 1.

RESULTS

Expression of the Transgene Correlates with Tumor Development. To test the oncogenic potential of the unactivated *neu* product in the mammary epithelium, a hybrid transcription unit comprising the MMTV promoter/enhancer and *neu* cDNA was microinjected into mouse zygotes. The MMTV/unactivated *neu* transgene is isogenic to the MMTV/activated *neu* described previously (14), except for the absence of the activating mutation in the transmembrane domain and the presence of a *Sal* I restriction endonuclease site between the *neu* cDNA and the SV40 polyadenylylation/splicing signals. A total of eight transgenic founders were generated carrying the MMTV/unactivated *neu* transgene. Of the eight strains, six passed the transgene to their progeny in a Mendelian fashion.

To assess the tissue specificity of transgene expression, 10 μg of total RNA derived from 10 different tissues isolated from both male and female carriers of the various founder strains was subjected to RNase protection with a transgene-specific probe comprising the SV40 polyadenylylation/splicing signals (pASV) (14). Representative results from these RNase protection experiments for the MMTV/*neu* strains are summarized in Table 1. Consistent with the observations made with other transgenic strains bearing MMTV/oncogenes, transgene expression was noted in mammary glands of female transgenic mice in five of six lines. Lower amounts of transgene transcript were detected in other tissues such as the salivary gland, spleen, thymus, and lung after longer exposure of the autoradiograms (Table 1).

Although low-level expression of *neu* in the mammary epithelium did not initially affect mammary gland function or development, focal mammary tumors began to appear in these strains at 4 months of age. Histological examination of the tumors revealed focal mammary adenocarcinomas surrounded by hyperplastic mammary epithelium (Fig. 1*B*). These tumors were composed of solid nests of pale intermediate cells that were morphologically identical to tumors associated with activated *neu* (25). Transplantation of the tumor cells into the mammary fat pads of syngeneic recipients resulted in the appearance of tumors, confirming their neoplastic potential. In our best characterized line, N#202, 50% of female carriers developed mammary tumors by 205 days (Fig. 1*A*). The appearance of tumors in this particular line was not strictly dependent on pregnancy, because virgin female transgenic mice also developed mammary tumors (data not shown). Furthermore, female transgenic mice derived from the other transgenic strains (N#169, N#510, N#721, and N#732) also developed tumors, albeit with later onset (Table 1). However, male MMTV/*neu* carriers did not exhibit any phenotypic abnormalities.

Because overexpression of *neu* (*ERBB2*) in human breast cancer has been implicated as an important step in tumor progression, we compared the level of transgene expression in the tumors and in the adjacent mammary epithelium. To this end, 10 μg of total RNA derived from mammary tumors and adjacent mammary epithelium isolated from female N#202 transgenic mice was hybridized with a radiolabeled transgene-specific probe (pASV) and subjected to RNase digestion (Fig. 2). To ensure that equal quantities of RNA were loaded, an *rpL32* antisense probe was also included in the hybridization reactions. Representative results for several sets of matched tumor and adjacent mammary epithelium for the N#202 line are shown in Fig. 2. Densitometric measurement of these autoradiograms revealed that many of the tumors ($n = 16$) expressed higher (10- to 50-fold) levels of transgene RNA than the adjacent mammary epithelium. However, several other matched sets of normal and tumor tissues ($n = 7$) expressed equivalent amounts of *neu* RNA (e.g., N#5741; Fig. 2). While there was some variation in transgene expression in the normal mammary epithelium, all mammary tumors examined expressed elevated levels of the *neu* transgene (Table 1).

To establish whether the elevated expression of transgene transcripts observed in the tumor tissues resulted in a corresponding increase in neu protein levels, Western analyses with neu-specific antibodies were conducted on protein extracts of normal and neoplastic tissues. The level of neu

Table 1. Transgene expression and onset of tumors in MMTV/unactivated *neu* mice

Line	Sex	Expression							Onset of tumor formation, days	% metastatic* tumors	Tumor type†
		M.gl.T	M.gl.N	Sal	L	SV	T	Ep			
N#169	F	+++	+	+	−				337 ($n = 4$)	50 ($n = 2$)	M.gl.Ad.
	M	−	+	+	−	−	+	+	NA	NA	No tumor
N#202	F	+++	+	+	+				205 ($n = 57$)	72 ($n = 41$)	M.gl.Ad.
	M	−	+	+	+	−	+	+	NA	NA	No tumor
N#204	F	−	−	−	−				NA	NA	No tumor
	M	−	−	−	−	−	−	−	NA	NA	No tumor
N#510	F	+++	+	+	−				367 ($n = 2$)	50 ($n = 1$)	M.gl.Ad.
	M	−	−	−	−	−	−	−	NA	NA	No tumor
N#721	F	+++	+	+	−				261 ($n = 21$)	11 ($n = 2$)	M.gl.Ad.
	M	−	+	+	−	+	−	+	NA	NA	No tumor
N#732	F	+++	+	+	−				268 ($n = 7$)	43 ($n = 3$)	M.gl.Ad.
	M	−	+	+	−	+	−	+	NA	NA	No tumor

RNase protection analysis was performed on 10 μg of total RNA isolated from a variety of organs from both male (M) and female (F) carriers derived from the MMTV/*neu* transgenic lines. The probe used in this analysis is directed against the SV40 component of the transgene and yields a 784-nucleotide protected fragment (see Fig. 2). Relative levels of transgene expression are indicated by − (not detected), + (low), or +++ (high). M.gl.T. refers to mammary gland tumor, whereas M.gl.N. represents normal adjacent epithelium. Other tissues examined for expression of the transgene included salivary glands (Sal), lung (L), seminal vesicles (SV), testes (T), and epididymis (Ep). NA, not applicable; *n*, number of animals analyzed.

*Percentage of tumor-bearing mice over 8 months of age possessing lung metastases.

†M.gl.Ad., mammary gland adenocarcinoma.

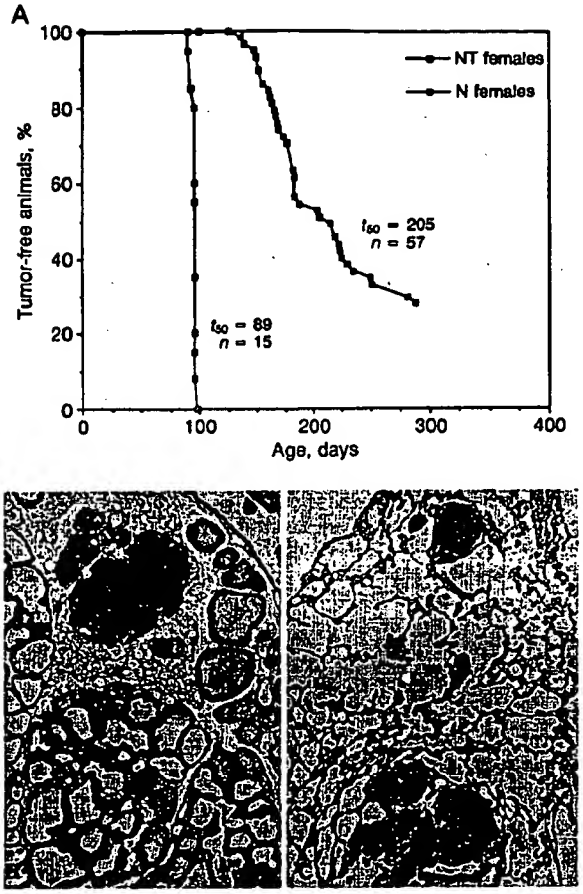

FIG. 1. Kinetics of tumor occurrence and histopathology of the MMTV/unactivated *neu* transgenic mice. (*A*) Comparison of the kinetics of tumor formation between female transgenic carriers bearing the MMTV/activated *neu* (NF line) (14) and females carrying the MMTV/unactivated *neu* construct N (N#202 line). The age at which 50% of mice were found to have tumors (t_{50}) and the number of mice examined (n) are indicated. (*B*) Photomicrograph of a hematoxylin/eosin-stained slide illustrating the expansile focus of solid tumor arising in the midst of a hyperplastic, dysplastic mammary gland (N#128, 210 days of age). Note that the dysplastic mammary cells lining dilated luminal spaces do not show lipid production. (×48.) (*C*) Photomicrograph of a hematoxylin/eosin-stained slide demonstrating a typical nest of mammary adenocarcinoma cells metastatic in the lung (N#202, 221 days of age). Note that the metastasis is in solid nests which resemble those found in the primary tumors such as those illustrated in *B*. (×48.)

protein in the tumors was higher than in the adjacent epithelium (Fig. 3*A*). Taken together, these observations suggest that elevated expression of *neu* may be an important step for tumorigenesis.

Mammary Gland-Specific Expression of *neu* Is Associated with the Induction of Metastatic Disease. Surprisingly, a high percentage of tumor-bearing MMTV/*neu* animals developed metastases to the lung (Fig. 1*C*). Histological examination of lung tissue in these affected animals revealed the presence of multiple foci of metastatic mammary adenocarcinoma lodged in pulmonary vessels (Fig. 1*C*). Like the primary mammary tumors, these metastatic lung tumors also expressed elevated levels of the *neu* transgene RNA (Fig. 2). The extent of metastatic involvement in these lines was particularly remarkable with respect to its penetrance. For example, in the N#202 line, 72% of the tumor-bearing mice that lived to an age of 8 months or older developed metastatic disease. Similar proportions of older tumor-bearing mice from the N#169, N#510, N#721, and N#732 lineages also developed metastatic disease (Table 1). Consistent with these observations, metastatic foci could also be detected in lung tissue after transplantation of the primary tumors into the fat pads of normal syngeneic recipients.

To confirm that the tumors detected in the lung were of mammary origin, RNase protection analyses with a probe specific to the mammary differentiation marker, β-casein, were performed on RNA derived from both primary and metastatic tumors (Fig. 2) (26). Both the primary mammary tumors and lung metastases derived from the N#202 line expressed moderate levels of β-casein RNA. However, normal lung tissue isolated from a nontransgenic female sibling did not express detectable amounts of β-casein RNA. Together with the histological observations, these results demonstrate that expression of unactivated *neu* in the mammary epithelium leads to the development of metastatic disease.

Induction of Mammary Tumors by *neu* Results in Elevated neu Tyrosine Kinase Activity. Because the transforming potential of the neu protein is closely correlated with its intrinsic tyrosine kinase activity, we were interested in measuring neu kinase activity in tumor and adjacent mammary epithelium derived from female animals of the N#202 line. To accomplish this, protein extracts derived from normal and tumor tissues were subjected to *in vitro* kinase assays using a monoclonal antibody directed against the rat neu protein. A prominent 185-kDa phosphorylated band was observed when extracts of mammary tumors and their derived metastases were assayed (Fig. 3*B*). By contrast, no comparable autophosphorylated species could be detected when extracts of nontransgenic control tissues or the adjacent mammary epithelium were assayed for kinase activity (Fig. 3*B*). Because neu protein could readily be detected in the mammary epithelium adjacent to the tumor tissue (Fig. 3*A*), these observations indicate that the *neu*-induced tumors possess higher neu-associated tyrosine kinase activity than the adjacent mammary epithelium.

DISCUSSION

Our observations provide direct evidence that expression of the protooncogenic form of *neu* results in a heritable development of metastatic mammary tumors. In five independent strains of MMTV/unactivated *neu* mice (N#202, N#169, N#510, N#721, and N#732), expression of the transgene resulted in the appearance of focal mammary adenocarcinomas which eventually metastasized to the lung. Tumorigenesis in these lines was correlated with elevated expression of the *neu* transgene and an increase in neu-associated tyrosine kinase activity. These observations support the hypothesis that elevated expression of *neu* can induce the production of metastatic mammary adenocarcinomas.

Overexpression of *neu* has been frequently observed in human primary breast cancers (10–13) and derived cell lines (1, 12, 27). In a large percentage of these human samples, overexpression of *neu* was associated with gene amplification. Consistent with these observations, RNase protection and Western analyses of tumor tissue (Figs. 2 and 3*A*) also revealed evidence of elevated expression of *neu* in mammary tumors. However, unlike human breast cancer samples, these tumors exhibited no evidence of amplification of the transgene or endogenous *neu* gene (data not shown). Conceivably, an increased transcription rate or an increase in mRNA stability of the transgene product could account for elevated *neu* expression. Indeed, several human mammary tumor cells express elevated levels of *neu* RNA in the absence of detectable gene amplification (27).

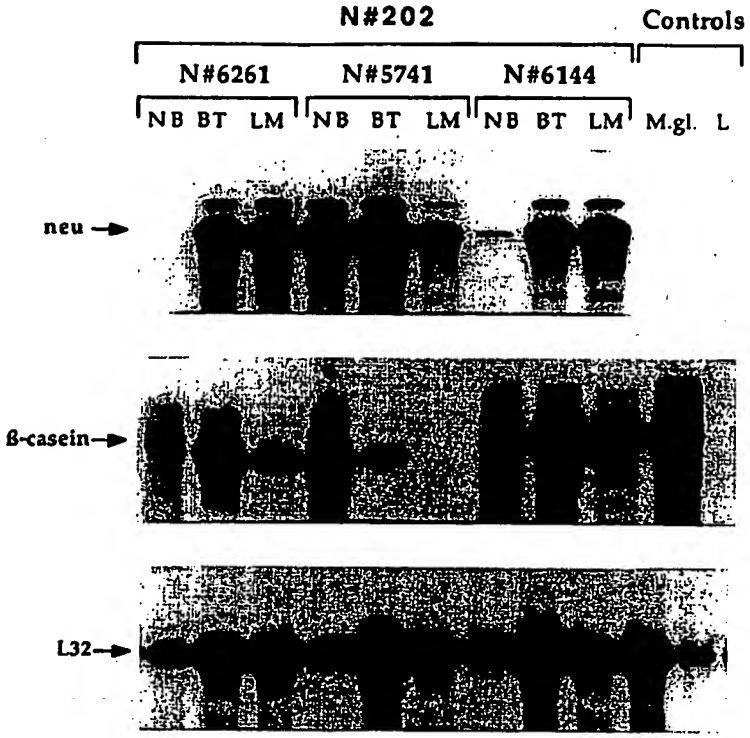

FIG. 2. RNase protection analysis of expression of the transgene RNA in tumor and adjacent mammary epithelium. RNA from control and transgenic tissues hybridized with probes directed to the transgene (neu), β-casein, and the *rpL32* ribosomal protein gene. The control tissues were isolated from lactating mammary glands (M.gl.) and lung (L) of normal female FVB mice. Transgenic tissues derived from multiparous female N#202 carriers (N#6261, 301 days of age; N#5741, 342 days of age; and, N#6144, 295 days of age) include primary mammary tumor (BT), adjacent mammary epithelium (NB), and lung metastases (LM). The 784-nucleotide protected fragment corresponds to the SV40 component of the transgene (neu), the 205-nucleotide protected fragment for β-casein, and the 278-nucleotide protected fragment for the *rpL32* control are indicated by arrows.

Previous studies have demonstrated that transgenic mice expressing the activated *neu* oncogene in the mammary epithelium also develop mammary tumors which are cytologically indistinguishable from those associated with the *neu* protooncogene (14, 15). However, malignant progression in strains carrying the MMTV/activated *neu* gene differs from the process observed here in the MMTV/unactivated *neu* transgenic mice. Mammary gland-specific expression of the activated *neu* oncogene in these strains was associated with the rapid and synchronous development of multifocal mammary tumors in both sexes (14). These studies suggested that expression of activated *neu* was sufficient for mammary tumorigenesis. By contrast, expression of unactivated *neu* is not sufficient, since mammary epithelium adjacent to tumors expresses appreciable levels of neu protein (Fig. 3*A*). It is conceivable that differences in the activity of the neu tyrosine kinase in transgenic mice expressing either the activated or the unactivated *neu* transgene account for this phenotypic variation. Consistent with this hypothesis is the observation that the *neu*-induced tumors possess higher tyrosine kinase activity than the adjacent mammary epithelium (Fig. 3*B*). Whereas the product of the activated *neu* transgene is constitutively active, the wild-type neu kinase may require additional events. For example, increased expression of neu receptor or its ligand could constitutively activate the neu kinase. Alternatively, neu kinase activity might be influenced by mutation or by the action of other cellular proteins. Consistent with the hypothesis that increased neu tyrosine kinase activity is required for tumor formation, a number of phosphotyrosine-containing proteins, including proteins of 185 kDa and 56 kDa, were specifically detected in tumor tissue by Western blot analyses with anti-phosphotyrosine

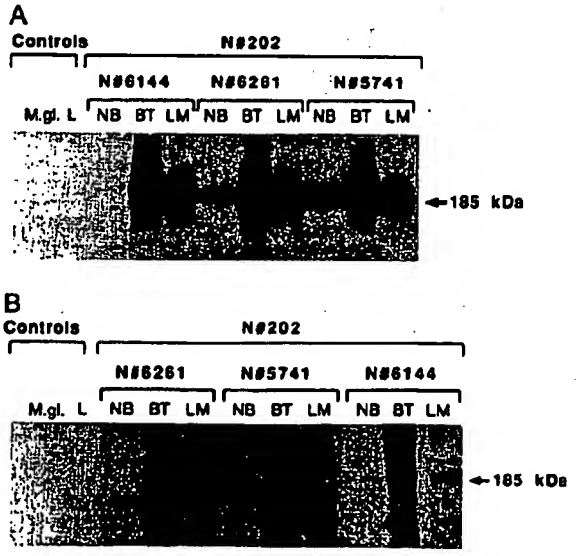

FIG. 3. Expression of neu protein and associated kinase activity in tumor and adjacent mammary epithelium. (*A*) Western analyses of control and transgenic tissues with a neu-specific monoclonal antibody. The control and transgenic tissues were isolated from the sources described in Fig. 2. The 185-kDa neu protein is indicated. (*B*) *In vitro* kinase activities of the same samples. Protein extracts were incubated with neu-specific monoclonal antibody, and the immunocomplexes were incubated with [γ-^{32}P]ATP. The 185-kDa phosphorylated neu species is indicated.

antibodies (data not shown). Because *in vitro* kinase analyses had demonstrated elevated neu kinase activity in tumor tissue (Fig. 3*B*) the 185-kDa protein very likely represents the autophosphorylated form of neu.

The unexpected finding that many of the older tumor-bearing *neu* transgenic animals developed pulmonary metastases may have important clinical implications. The observation that overexpression of *neu* in human breast cancer is associated with poor clinical outcome in node-negative women (28, 29) and the results of these transgenic experiments suggest that overexpression of *neu* can confer an enhanced metastatic potential upon the mammary tumor cell. Pulmonary metastases were also observed in transgenic strains carrying the MMTV/activated *neu* transgene (14). However, metastasis in these mice was relatively infrequent. Because these activated *neu* tumors involve the entire mammary epithelium and thus considerably shorten the animals' survival, it is conceivable that the further steps necessary for metastatic progression do not have sufficient time to occur in these MMTV/activated *neu* mice. Consistent with this notion, metastasis in the MMTV/unactivated *neu* lines is observed only in mice that have carried mammary tumors for several months (Table 1). Determination of the mechanism by which the neu kinase is involved in the induction of metastatic disease awaits further analysis.

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2 The NCI In Vitro Anticancer Drug Discovery Screen

Concept, Implementation, and Operation, 1985–1995

Michael R. Boyd, MD, PhD

CONTENTS

1. INTRODUCTION

1.1. The Background

Since 1955, the US National Cancer Institute (NCI) has provided screening support to cancer researchers worldwide. Until 1985, the NCI screening program and the selection of compounds for further preclinical and clinical development under NCI auspices had relied predominantly on the in vivo L1210 and P388 murine leukemias and certain other transplantable tumor models (*1*). From 1975–1985, the in vivo P388 mouse leukemia model was used almost exclusively as the initial or primary screen. With few exceptions, agents that showed minimal or no activity in the P388 system were not selected by the NCI for further evaluation in other tumor models or alternative screens. Most of the available clinical anticancer agents are active in the P388 system; however, most were discovered prior to 1975 or by observations initially in test systems other than the NCI-operated P388 primary screen.

1.2. The Concept

In June of 1984, the author presented to the NCI Division of Cancer Treatment's Board of Scientific Counselors (BSC) a preliminary concept of a so-called disease-oriented in vitro primary anticancer drug screen as a potential replacement to the P388 in vivo primary screen. Although the new concept was greeted initially with limited enthusiasm, the presenter nonetheless was encouraged to return to the subsequent fall meeting of the BSC with a more fully developed concept for further review and discussion. The new screening model that was proposed to the Board at the

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October 1984 meeting (*2,3*) comprised the essence of the operational screen, which was formally launched (*4–11*) in 1990, and likewise which is embodied in the present-day screen (*12,13*).

The main focus of this chapter is the 5-yr interval, 1985–1990, during which the author and staff of the NCI Developmental Therapeutics Program (DTP) were intently engaged in the effort to convert concept to reality in the form of a new operational primary screen. Having fortunately seen that goal accomplished in 1990, the author requested and was granted reassignment to full-time duty in the NCI intramural research program. This afforded a unique opportunity both to explore research applications of the new screen more directly and to observe and reflect on, from a more personal perspective, the subsequent 5-yr evolution of the in vitro service screen and its in vivo counterpart. Other chapters in the present volume provide both historical and current details and perspectives of the NCI in vivo antitumor testing program (J. Plowman et al, Chapter 6) as well as the overall drug discovery and development organization, strategies and progress of the NCI Developmental Therapeutics Program (E. Sausville, Chapter 11).

1.3. The Debate and Decision

Following the author's October 1984 presentation of the new screen concept, the BSC expressed sufficient interest to name a subcommittee to meet separately with DTP staff to consider the concept in further detail. Subsequently we and the subcommittee under the chairmanship of Mortimer M. Elkind joined in the planning and organization of a workshop entitled "Disease-Oriented Antitumor Drug Discovery and Development." That workshop, held in Bethesda, MD on January 9–10, 1985, provided a forum for extensive discussion and debate concerning the justification or otherwise of such a proposed radical departure from the existing NCI drug screening paradigms. Participants in the workshop, in addition to DTP and other NCI staff, represented a wide cross-section of experts from academia and industry, both from the US and abroad (*see* Appendix A). The debate, well documented by a verbatim transcript (*14*), was vigorous, but did not reveal any clear unanimity of opinion either regarding the shortcomings of existing screens or alternative screening models or strategies. Nevertheless, there was a consensus that new screening and discovery strategies in general merited consideration, particularly given the prevailing dearth of effective therapeutic agents for the majority of human solid tumors. Following the workshop, the author again presented the new screen concept to the BSC at the February 1985 meeting (*4*), for final review, further debate, and vote. The Board voted affirmatively, recommending that an initial 2-yr exploratory effort be pursued in parallel to a continuing, although substantially downsized, P388 in vivo screen (*4*). The board additionally approved the author's proposed concurrent DTP-NCI implementation of a comprehensive new natural products collection, extraction, and repository program (*4*).

In mid-1985, DTP staff began the phase-down and eventual termination of the in vivo P388 primary screen, the human tumor colony-forming assay (HTCFA) screen (*15*), and the "tumor panel" secondary screens (*1*). Simultaneously, we launched the pilot program to explore the feasibility of the proposed new in vitro primary screening model. The screen would employ a diverse panel of human tumor cell lines organized into subpanels representing major tumor types. Compounds would be tested over a

wide range of concentrations for cytotoxic or growth-inhibitory effects against each cell line comprising the panel. A secondary stage of screening on selected compounds would be performed in vivo in xenograft models using a subset of the cell lines found to be sensitive in the in vitro screen.

Although simple in concept, the development and implementation of the new in vitro NCI primary screen presented unprecedented challenges. Nevertheless, the feasibility of the pilot screen was firmly established by mid-1989, and in April 1990 the screen was established in fully operational status. Beginning in 1990, samples were screened at a rate of approx 20,000/yr, with the input divided about equally between pure compounds submitted to the NCI, and extracts or fractions thereof originating primarily from the NCI natural products repository. In its first operational configuration, the cell line panel consisted of a total of 60 human tumor cell lines arranged in subpanels representing diverse histologies.

2. IMPLEMENTATION 1985–1990

2.1. Overview and Oversight

Efforts to establish the feasibility of the proposed new in vitro primary screen were focused initially on three main fronts: investigation of various alternative assays of in vitro drug sensitivity (*16–23*); development of the cell line panel (*23–25*); and information technology (*6,26,27*). The technical challenges to implementation of such a screen, requiring on the order of 10–20 million individual cell culture assays/yr to achieve the scope of operations envisaged, were daunting. Also, many critical choices had to be made with respect to the design principles of the screening model, which would, in turn, have profound impact on screening operational logistics as well as the nature of the data output and potential utility thereof. To assist us in making such critical decisions, as well as to provide in-depth, regular oversight of implementation of the new program, we organized an internationally comprised, external "Ad Hoc Review Committee for the NCI In Vitro/In Vivo Disease-Oriented Screening Project" under the chairmanship of Kenneth R. Harrap. Participants (non-NCI) in one or more meetings of that key committee (the "Harrap committee") during its 1985–1990 existence are named in Appendix B. The Harrap committee, or a subcommittee thereof, met at least once annually with NCI staff for detailed discussions, debate, and critique of the new program. The 1987 meeting of the Harrap committee was combined with a second workshop entitled "Selection Characterization, and Quality Control of Human Tumor Cell-Lines for the NCI's New Drug Screening Program," jointly organized and held in Bethesda, MD on May 27–28, 1987. Participants in that meeting are named in Appendix C. Verbatim transcripts of all these meetings (*14,28–32*) provide interesting documentation of the progress, as well as the challenging technical problems encountered, during the 1985–1990 period. In addition to the Harrap committee reviews, the development of the new screen was also reviewed periodically during regular meetings of the full membership of the BSC and likewise by the National Cancer Advisory Board (NCAB).

2.2. In Vitro Microculture Assays of Cell Growth and Viability

Three alternative assays for cellular growth and viability for possible use in the new primary screen were extensively investigated (*16–23*). Two were metabolic assays

(*16,18*); in both, the cellular reduction of a colorless tetrazolium salt (MTT or XTT) yielded a colored formazan in proportion to viable cell number. The formazans could be measured conveniently in an automated colorimeter.

The development of the XTT tetrazolium assay (*18*) was stimulated by the desire to simplify further the MTT procedure by eliminating an aspiration/solubilization step; the reduction of MTT yielded an insoluble formazan, which had to be dissolved in dimethylsulfoxide prior to colorimetry. In contrast, the XTT reagent (*17*) was metabolized by viable cells directly to a water-soluble formazan, allowing the immediate reading of optical density in the culture wells without further processing. Although simple and convenient, the XTT procedure gave relatively high background readings (low "signal-to-noise" ratio). XTT also shared with MTT the feature of an unstable (i.e., time critical) end point, compromising the potential use of either of the tetrazolium assays in a high-flux antitumor screen employing a large panel of cell lines.

Although the XTT tetrazolium assay ultimately was not adopted for the anticancer screen, it did prove to have an immensely valuable application in the DTP-NCI high-flux anti-human immunodeficiency virus (HIV) drug discovery screen. The concept and development of the anti-HIV screen was first proposed by the author to NCI management in November 1986, was subsequently presented to and formally approved by the BSC in February 1987, and was pursued thereafter by DTP in parallel to the anticancer screen (*33,34*). We also organized a separate external Ad Hoc Advisory Committee, initially under the chairmanship of Dani P. Bolognasi and later under William M. Mitchell, to provide critique and oversight of development of the anti-HIV screen. Verbatim transcripts of the major three meetings of that committee during 1987–1990 were similarly recorded (*35–37*); names of the committee members are available in the transcripts. This committee met concurrently with the Harrap committee at its 1989 meeting.

For the anticancer screen application, there were two especially troublesome problems encountered with the tetrazolium assays that eventually prompted the development of a third alternative microculture assay method. For either MTT or XTT, tetrazolium reduction was dependent on the cellular generation of NADH and NADPH. This raised concern about the influence of glucose concentration on the formation of the colored tetrazolium formazan, which was measured colorimetrically as an estimate of cellular growth or viability. Studies with MTT indicated that a progressive reduction in MTT specific activity (MTT formazan formed/μg cell protein), which was observed during the course of a typical 7-d assay, was paralleled by a progressively decreasing glucose concentration (*19*). For XTT, there was a further problem resulting from the additional requirement of an electron transfer reagent, phenazine methylsulfate (PMS), to promote adequate cellular reduction of the tetrazolium. With XTT/PMS, variations in pH of the standard growth medium (RPMI-1640), typically caused by temporary removal of culture plates from the relatively high 5% CO_2 incubator environment, resulted in occasional formation of a crystalline material causing erratic optical density measurements. Crystal formation occurred in the pH range of 7.0–9.0 and could be attributed to reaction of PMS with glutathione (*19*).

In an attempt to eliminate the pH instability problem, a new culture medium was developed (*20*). The medium had a stable physiological pH of 7.4 at normal atmospheric levels of CO_2 and derived its buffering capacity primarily from β-glycerophosphate. The new medium was optimized to facilitate growth in atmospheric CO_2 by

inclusion of biotin, L-asparagine, pyruvate, and oxaloacetate for metabolic stimulation of intracellular CO_2 production. With either the MTT assay or a nontetrazolium assay (described below), similar dose-response curves were obtained for various standard anticancer drugs against cell cultures maintained either in the new medium (PDRG basal growth medium) under ambient CO_2 or in RPMI-1640 under a 5% CO_2 environment. However, a decision was subsequently made, consistent with a specific recommendation of the Harrap committee, not to incorporate the PDRG medium into the new screen, but rather to consider an alternative end point assay that was not as dependent on the particular CO_2 environment.

In an attempt to identify a suitable, nontetrazolium assay for use in the in vitro primary drug screen, a series of protein and biomass stains were investigated (*21*). These included anionic dyes that bound to the basic amino acid residues of proteins, as well as cationic dyes that bound to the negative, fixed charges of biological macromolecules. Of all the reagents tested, sulforhodamine B (SRB) gave the best combination of stain intensity, signal-to-noise ratio, and linearity with cell number. SRB is a bright pink anionic dye that, in dilute acetic acid, binds electrostatically to the basic amino acids of TCA-fixed cells.

2.3. *Selection of Assay Parameters and Methodology*

Under in vitro assay conditions, exposure to an antitumor agent may decrease the number of viable tumor cells by direct cell killing or by simply decreasing the rate of cellular proliferation. Many in vitro assays of drug sensitivity typically employ relatively low initial cell inoculation densities (e.g., a few hundred cells/well) followed by relatively long continuous drug exposure times (e.g., 6–7 d or considerably longer than the doubling times of many tumor lines). Such a selection of assay parameters, although favoring the detection of antiproliferative effects (i.e., growth inhibition), might, however, obscure otherwise potentially interesting patterns of differential cytotoxicity (e.g., net cell killing). Moreover, with an antiproliferative or growth-inhibition end point, cell lines with very short doubling times (e.g., leukemias) might appear hypersensitive in comparison to more slowly growing tumor lines (e.g., from solid tumors). Additionally, potential problems of nutrient deprivation, as well as practical limitations on the use of pulse drug exposures, might, in the course of an assay, necessitate removal and replacement of medium. On the other hand, a longer assay duration might facilitate the detection of activity of relatively insoluble compounds or active trace constituents in mixtures or extracts. Furthermore, the longer assay format might be essential for detection of agents that required several cell cycles for expression of lethal drug effects.

An alternative selection of assay parameters was considered in order to enhance the screen's ability to discern interesting differences in net cell killing (i.e., actual reduction of biomass) among the sensitive panel lines. This required the use of a relatively large initial cell inoculum (e.g., 20,000 cells/well), and a relatively short drug exposure/incubation time (e.g., 1–2 d). Optimal exploitation of this format required a high level of sensitivity and reproducibility of the assay methodology, and the capability to measure reliably the initial viable cell densities ("t_0" values) just prior to drug introduction.

There were reasonable arguments for and against selection of either of these two alternative sets of assay parameters, or some compromise in between. Indeed, we

extensively investigated the impact of these parameters on the screen's performance and, not surprisingly, found that certain kinds of compounds yielded results that contrasted greatly, depending on the particular choice of assay parameters. However, for purposes of further studies with the pilot-scale screen, as well as for initiation of the full-scale screen, the high-cell-inoculum/short-assay protocol was selected for rountine use. This selection was based principally on the desire to minimize the effects of variable doubling times of the diverse cell lines in the panel, to optimize the chances of detection of cell-line-specific or subpanel-specific cytotoxins, and to minimize the chances of obscuring such activities by nonspecific antiproliferative effects. This choice of assay parameters was also emphatically endorsed by the Harrap committee, after much discussion, debate, and extensive review of the relevant available experimental data.

Given the above decisions concerning assay parameters, the optimal choice of a tetrazolium assay (e.g., MTT or XTT) vs the SRB assay had to be determined for the desired application to a large-scale screening operation employing simultaneously many diverse tumor lines. Pilot-screening studies (*22,23*) were performed on a common set of compounds using both MTT and SRB, along with the selected assay parameters. Under the experimental conditions employed and within the limits of the data analyses applied, the assays gave quite comparable results. However, the SRB assay had important practical advantages for large-scale screening. Although the SRB procedure was more labor-intensive (e.g., required multiple washing steps), it had the distinct advantage of a stable end point (i.e., not time critical, in contrast to either of the tetrazolium assays). Screening capacity, reproducibility, and quality control all appeared to be markedly enhanced by adoption of the SRB for the primary screen (*23*). Therefore, the SRB assay was used subsequently for all routine screening operations.

2.4. Cell Line Panel

The initial panel incorporated a total of 60 different human tumor cell lines derived from seven cancer types, including lung, colon, melanoma, renal, ovarian, brain, and leukemia. Selection of lines for inclusion in the panel required that they adequately met minimal quality-assurance criteria (testing for mycoplasma, MAP, human isoenzyme, karyology, in vivo tumorigenicity), that they were adaptable to a single growth medium, and that they showed reproducible profiles for growth and drug sensitivity. Mass stocks of each of the lines were prepared and cryopreserved; these stocks provided the reservoir for replacement of the corresponding lines used for drug screening after no more than 20 passages in the screening laboratory (*6,23,24*).

Although many of the lines were well known and had been widely used in research, the clinical histories and/or original tumor pathologies of many of the lines were incomplete or unavailable. All cell lines in the interim panel were nevertheless subjected to detailed, specialized characterizations (e.g., histopathology, ultrastructure, immunocytochemistry) to verify or determine tissue and tumor type (*25*). Moreover, parallel projects were launched for the acquisition of better and more diverse candidate cell lines, and for the development of new lines directly from surgical specimens or from nude mouse xenografts for which the corresponding clinical backgrounds were more complete. Special focus was placed on major cancer types (e.g., breast and prostate) that were not represented in the initial panel owing to unavailability of suitable lines.

2.5. Pilot Screening Operations: Standardization and Reproducibility

A pilot-screening operation (*6,23*) was initiated in which the panel lines were inoculated onto a series of standard 96-well microtiter plates on day 0, in the majority of cases at 20,000 cells/well, and then preincubated in absence of drug for 24 h. Test agents were then added in five, 10-fold dilutions starting from the highest soluble concentration, and incubated for a further 48 h. Following this, the cells were fixed *in situ*, washed, and dried. SRB was added, followed by further washing and drying of the stained, adherent cell mass. The bound stain was solubilized and measured spectrophotometrically on automated plate readers interfaced with personal computers, which, in turn, were interfaced to a central computer.

A series of approx 170 known compounds, comprising commercially marketed (NDA-approved) anticancer agents, investigational (investigational new drug application [INDA]-approved) anticancer agents, and other candidate antitumor agents (compounds previously approved by the NCI Decision Network Committee for preclinical development based on activities in prior screens) were selected for pilot screening studies (*6*). The repetitive screening of these prototype "standard agents" was aimed at providing a suitable data base from which a variety of novel approaches to data display and analysis could be explored. The "standard agent data base" was also the basis for calibration and standardization of the screen, for the assessment of reproducibility of the screening data, and for the development of procedures for quality-control monitoring (*6,27*).

2.6. Information Technology

Facilitating the above analyses were the development of the COMPARE pattern-recognition methodology and the mean-graph display, which supported both visual and automated analyses of the differential activity profiles of agents tested against the 60-cell panel (*6,12,13,26,27*). The mean-graph profiles of standard agents were highly reproducible over time; for example, the characteristic mean-graph profile of a given standard agent could be shown by COMPARE to be highly correlated among separate screening runs of the same compound over many months (*6,27*).

2.7. Review and Recommendation to Operational Status

In November of 1989, a pivotal review meeting (*5,32*) was held at the NCI-Frederick Cancer Research and Development Center in Frederick, MD, the site of the newly constructed screening facilities. The full current memberships of the Harrap committee and an additional ad hoc advisory subcommittee for the natural products program, the BSC, the NCAB, and the President's Cancer Panel were invited to review jointly in detail the progress of implementation of the in vitro screen, and to provide recommendations for further directions. The resulting consensus recommendation was that the feasibility, reproducibility, and calibration of the screen were sufficiently established that full-scale operation should be formally initiated as soon as possible (*5*). DTP staff responded accordingly, and full operational status of the screen was established shortly thereafter. Table 1 summarizes the assay protocol and parameters for the operational screen as launched in 1990. Table 2 summarizes some of the pertinent screening laboratory operations and logistics. The annual operational costs of the new in vitro

Table 1
Assay Protocol and Parameters for the NCI In Vitro Antitumor Screen as Initiated in 1990[a]

Cell line panel
60 Lines total
7 Subpanels initially (lung, colon, renal, ovary, melanoma, brain, leukemia)
Lines used at ≤ 20 passages from master stock
Culture medium
RPMI 1640
5% Serum
Cell inoculation densities: 5000–40,000 cells/well (96-well microtiter plate)
Preincubation: 24 h (no drug)
Sample dilutions
Routinely 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and $10^{-8}M$ or as specified
Duplicates performed at all concentrations
T_o and "no-drug" controls included
Drug incubation: 48 h
End point assay: Sulforhodamine B protein stain

[a]Further details are available in refs. (*6,21,23,25*).

primary screen were budgeted at approx $3–4 million/yr, or approximately one-third the operational costs of the P388 in vivo primary screen that it replaced.

3. OPERATION 1990–1995

3.1. Routine Screening Operations

For routine screening as initiated in 1990, each sample was tested in the 2-d, continuous-drug-exposure protocol using five, $\log_{10}$-spaced concentrations starting at an upper limit of $10^{-4}M$ (or 100 μg/mL for natural product extracts or fractions thereof) against all of the current 60 cell lines comprising the panel. Most crude extracts were initially "prescreened" using only a single concentration (100 μg/mL) against the entire 60-cell-line panel; extracts that produced ≥ 50% net cell killing of ≥ 3 of the panel lines were routinely selected for testing in the full screen.

Details of the particular cell lines comprising the original panel, and the individual inoculation densities and other assay features used initially in routine screening operations were as published (*23,25*). From December 1, 1992, a modified panel, in which 10 of the original cell lines were replaced by a selection of breast and prostate cancer lines, was employed. Details of the replacement cell lines, inoculation densities, and other aspects of routine screening operations as of end of 1995 were as published (*13*).

The testing of a sample in the full 60-cell-line screen yields a corresponding set of 60 dose–response curves. Figure 1A–D illustrates four contrasting sets of composite dose–response curves. In Fig. 1A, the particular test compound had essentially no effect on the growth or viability of any of the 60 cell lines. Figure 1B shows the effect

Table 2
Primary Screening Laboratory Operations and Logistics as Initiated in 1990[a]

Laboratory
8051 ft² total space
2 Floors
4 General support modules
20 Screening modules
50 Laminar-flow hoods
Staffing
44 Technicians
2 Senior Supervisors (Ph.D.)
Cell inoculations/drug additions
6 Lines assigned per technician
3 Lines/2 compounds/96-well plate
Colorimetric end-point determinations
10 Plates/compound
4000 Plates/wk
12 Automated plate readers
Quality control
Manual
Automated
Computer support: 20 in-lab PCs networked to central computer
Calibration/standardization of screen
Daily standards
Monthly standards
Standard agent database

[a]Further details are available in refs. (*6,23,27*).

of a test compound that was cytotoxic, although with essentially equivalent potency to all of the panel lines. Neither of these screening results is particularly useful. In contrast, Fig. 1C illustrates results from a compound showing pronounced cytotoxicity, although with considerably divergent potencies against the individual cell lines. Screening profiles, as exemplified by Fig. 1C, which manifest "differential" growth inhibition and/or cytotoxicity have been of particular interest as the basis for research applications of the screen, as well as for the selection and prioritization of compounds for in vivo evaluation. Figure 1D shows the characteristic dose-response composite profile from a highly potent natural product, dolastatin 10 (Fig. 2; *see also* refs. *38,39*); there appears to be a marked degree of differential growth inhibition and/or cytotoxicity among the various panel lines. However, for any given line, the inhibitory effect of the compound, within the tested concentration range, is not concentration-dependent. A more detailed analysis and explanation of the basis for this type of profile is provided elsewhere (*13*). Critical to any research application of the screen is the remarkably high degree of reproducibility of the screening profile of a given compound tested repetitively over time (*6,13,27*).

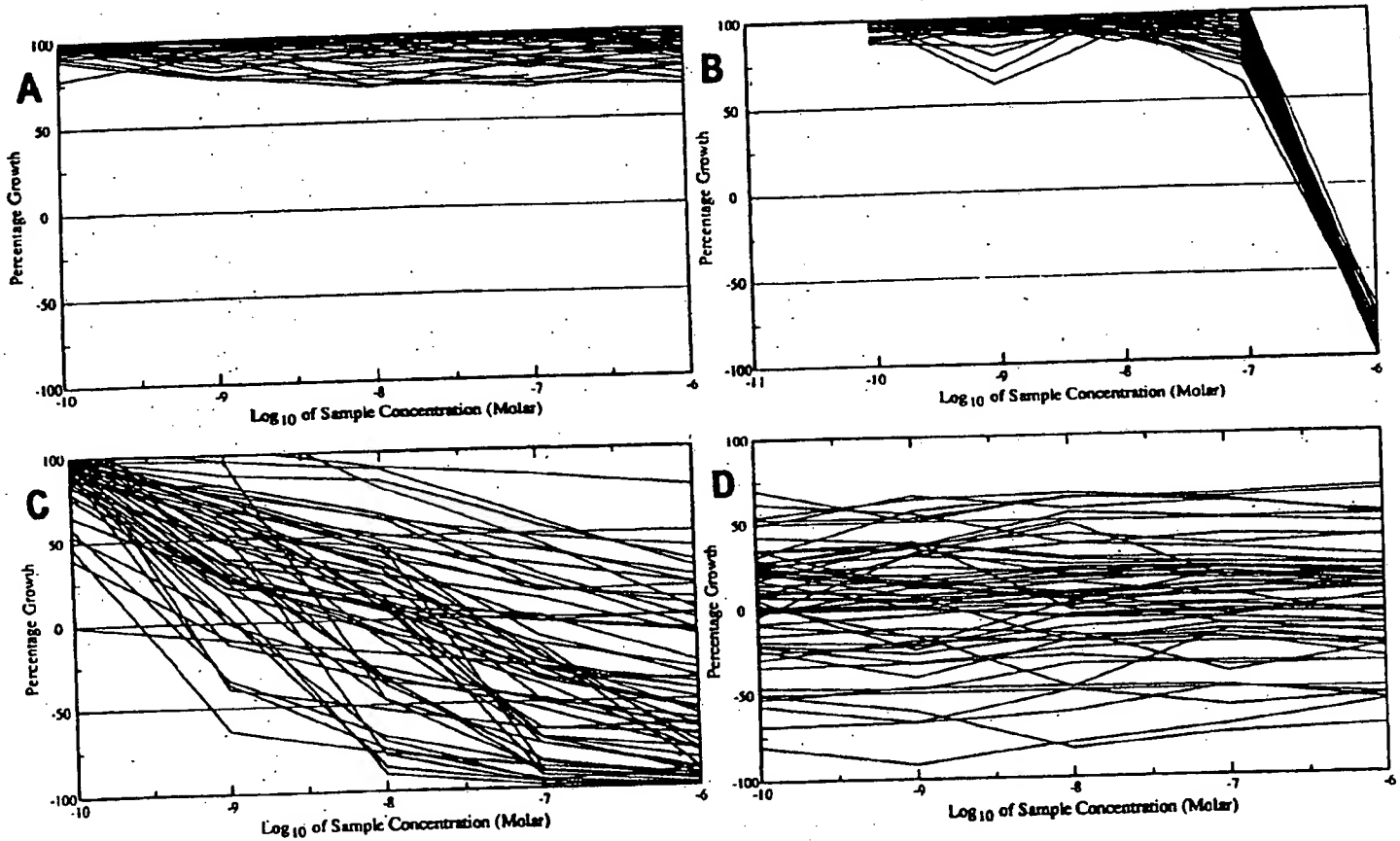

Fig. 1. Examples of composite 60-cell dose–response profiles of various compounds tested in the NCI in vitro screen. Composite **A** is from the screening of a compound that produced little or no growth inhibition or cytotoxicity to any of the cell lines. Composite **B** is from the screening of a compound showing general ("nondifferential") cytotoxicity at the highest tested concentration. Composite **C** shows results of screening of a compound that produced markedly "differential" cytotoxicity among the panel cell lines. Composite **D** illustrates another type of differential activity profile, in which there is no apparent dose dependence of the effects within the tested concentration range of the particular compound.

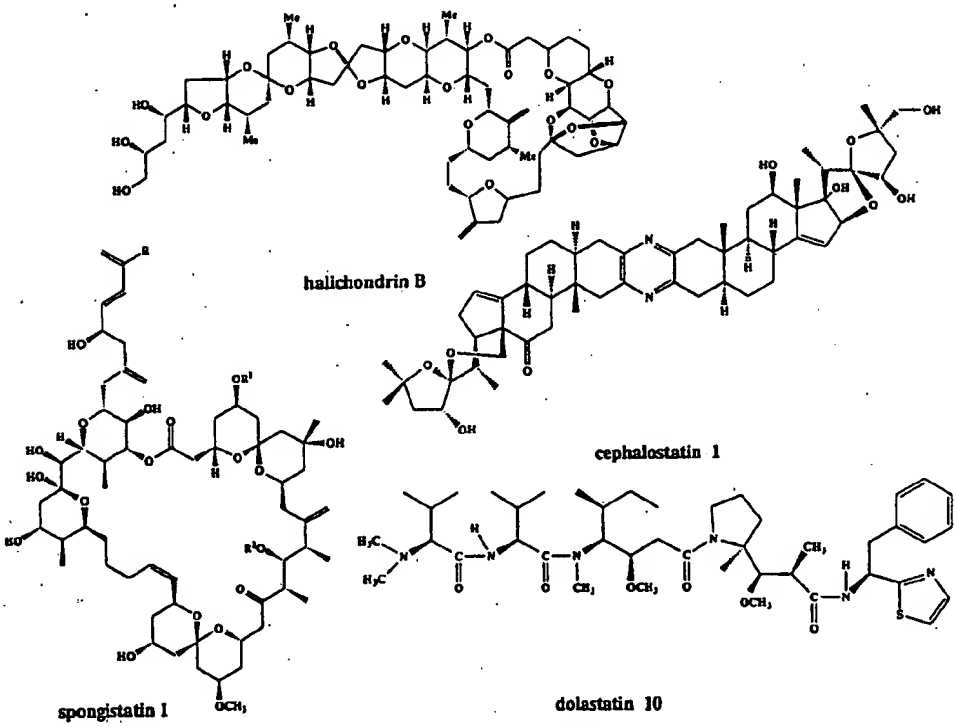

Fig. 2. Structures of some novel antitumor leads elucidated from marine natural products. Details of the initial elucidation of these challenging structures by the original investigators can be found in refs. (*38, 45–47, 54*). Samples of the compounds were provided by George R. Pettit for study in the NCI screen. Figure 1D is the composite dose-response profile obtained from testing of dolastatin 10. The composite screening profiles of spongistatin 1 and halichondrin B (not shown) closely resembled that of dolastatin 10 (Figure 1D). Figure 1C is the composite dose-response profile from testing of cephalostatin 1 in the in vitro screen.

3.2. Research Applications

NCI staff, collaborators, and others have been exploring diverse data analysis strategies and methods with data generated by the in vitro screen. Reviews and other publications describing such studies are available (*6,12,13,40–44,48*). As discussed recently (*13*), the appealingly simple mean-graph and COMPARE analysis methodologies provide useful support for a number of research applications. Such applications encompass the discovery of new members of known mechanistic classes. For example, the mean-graph screening profiles (not shown) of halichondrin B (*45,46*) and spongistatin 1 (*47*) (Fig. 2) were revealed by COMPARE to resemble closely the mean-graph profile (not shown) of dolastatin 10 (*38,39*) (Fig. 2) and other known members (e.g., vinca alkaloids, taxol, rhizoxin, maytansine) of the general class comprising tubulin-interactive antimitotics (*48*). Followup biochemical studies (*49,50*) confirmed the general antimitotic mechanism anticipated from the initial evaluation of halichondrin B and spongistatin 1 in the 60-cell screen.

The counterpoint to discovery of new members of known mechanistic classes is the discovery of new antitumor mechanistic classes and new members therein. Such application of the 60-cell screen is exemplified by studies of 9-methoxy-N^2-methylellipticinium acetate (MMEA) and related ellipticinium derivatives. MMEA produced an unprecedented screening profile, in which the brain tumor cell line subpanel showed consistently higher sensitivity to MMEA cytotoxicity than did other lines comprising the panel (*6,51*). Subsequent studies revealed a high correlation between uptake and accumulation of MMEA, and/or metabolite(s) thereof, and MMEA cytotoxicity in the sensitive brain tumor cell lines (*52*). Both uptake and cytotoxicity of MMEA were blocked by reserpine. Other experiments further suggested a resemblance of the MMEA transporter in the brain tumor lines to a constitutive biogenic amine transport process characteristic of certain glial elements of normal brain (*52*). Recent in vivo studies of the 9-chloro analog of MMEA have shown evidence of in vivo antitumor activity against an intracranially implanted brain tumor cell line (*53*).

Another novel lead that shows an unprecedented profile in the 60-cell-line screen is cephalostatin 1 (*54,55*) (Fig. 2). Figure 1C is from the screening of this compound. Figure 3 shows the GI_{50}, TGI, and LC_{50} mean graphs, constructed from the data of Fig. 1C, and defined in detail elsewhere (*13*). COMPARE analyses were performed, using described procedures (*13*), with the mean-graph profiles of cephalostatin 1 as the "seed" against a screening data base from approx 40,000 structurally diverse compounds. When the profiles were ranked in order of degree of similarity to the seed, the top-ranking 13 profiles were all found to be derived from prior tests of cephalostatin 1 or other closely related members of the cephalostatin series (*55*). On the other hand, the characteristic screening profile of the cephalostatins did not show comparable correlations to any member(s) of the standard agent data base, suggesting that the differential cytotoxicity of this lead derives from an unprecedented, but as yet undefined, mechanism of action.

Another important research application has become apparent from studies of structure–activity relationships (SAR) and chemical analog synthesis. In these applications, the NCI in vitro screen provides an opportunity for lead optimization based on the feedback of both quantitative and qualitative biologic data. For example, members of a chemically related series can be compared not only with respect to relative potencies, but also with respect to the degree to which they do or do not retain the desired cell line specificity or subpanel activity of the lead compound. Research applications of this nature are illustrated by recent studies (*51*) with the aforementioned ellipticinium series and also by SAR investigations related to the novel antitumor lead, halomon (*56,57*).

An exploratory research application of the screen has been pursued intramurally at NCI for the selection and bioassay-guided fractionation of natural product extracts from the NCI repository. The screen has been used either to select or, alternatively, to eliminate from further consideration extracts having screening profiles either similar to or distinctly different from any known standard agent or mechanistic class. Bioassay support for fractionation has employed either the full 60-cell screen or one or a few individual cell lines selected on the basis of the 60-cell screening profile. As of end of 1995, approx 80,000 extracts had been screened against the 60-cell panel, however, the majority of these were only in the single-concentration "prescreen" protocol. Extracts failing the initial prescreen-select criterion were not subjected to further

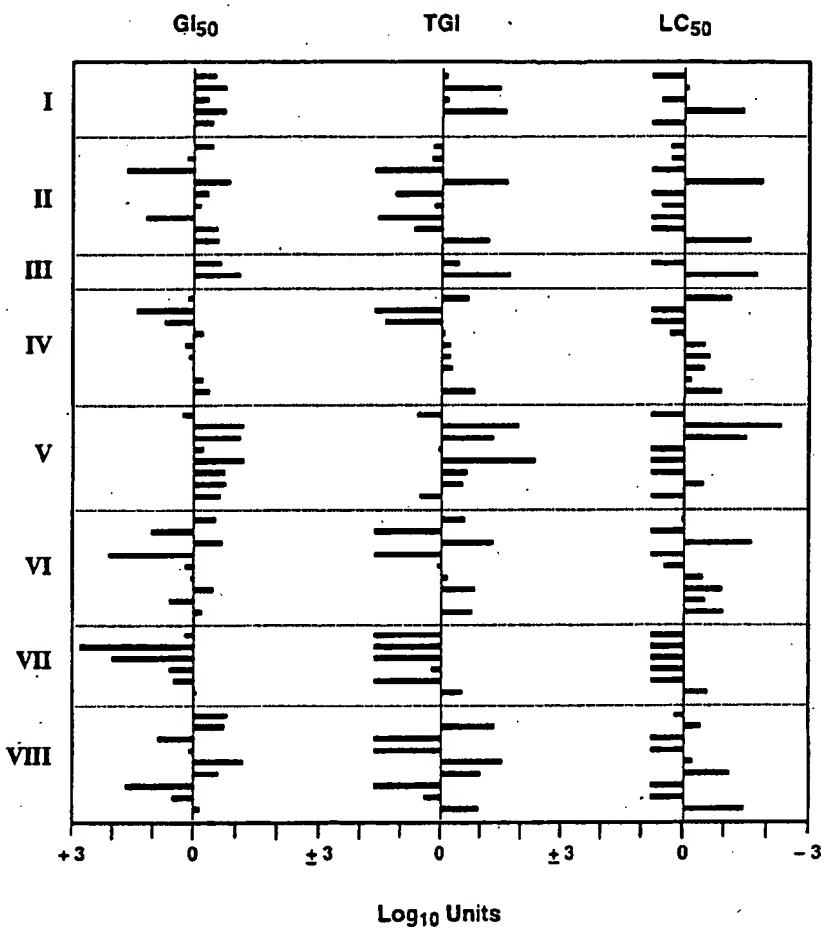

Fig. 3. GI_{50}, TGI, and LC_{50} mean graphs for cephalostatin 1 constructed from the data illustrated in Fig. 1C. Response parameter definitions, methods of constructions and interpretation of mean graphs have been reviewed recently in detail elswhere (*see* ref. *13*). The tumor cell line subpanels are identified as follows: I (leukemia); II (lung, nonsmall-cell); III (lung, small-cell); IV (colon); V (brain); VI (melanoma); VII (ovary); VIII (kidney). These mean-graph "fingerprints" can be shown by pattern-recognition analyses (e.g., *see* methods of COMPARE analyses also reviewed recently in ref. *13,44*) to be highly correlated with those of other cephalostatins; in contrast, they were not similarly correlated with any of the "standard agents" (*6,13*).

testing; as a result, the data base of natural products screening using the full, five-concentration assay against the 60-cell panel is not nearly as extensive as the data base derived from the full screen evaluation of pure compounds. Nevertheless, several hundred extracts having novel screening profiles were identified, and studies initiated to isolate, identify, and characterize further the individual active constituents.

There are other emerging research applications of the NCI in vitro screen aimed at exploiting advances in knowledge of tumor biology and the molecular genetics of cancer. For example, experimental measurements of the differential expression in the panel cell lines of potential cell growth regulatory and/or drug sensitivity or resistance

determinants are being used to construct hypothetical mean-graph profiles, which are then used to search the available data bases for compounds that produce actual screening profiles similar to the desired hypothetical one(s). For instance, a hypothetical mean-graph fingerprint constructed from quantitative expression values for the *mdr*-1/P-glycoprotein in each of the panel cell lines was used as the seed for COMPARE analyses (*58*). A series of compounds was thereby identified having screening profiles highly correlated with the constructed probe. Subsequent biochemical analyses confirmed that the selected compounds were indeed substrates for the P-glycoprotein. In a related study (*59*), comparably high correlations were found for the same compounds with respect to a probe constructed of rhodamine efflux values, which are functional assay counterparts of *mdr*-1 expression. NCI staff and collaborators are exploring similar research strategies with numerous other potential sensitivity or resistance determinants, such as oncogene or tumor suppressor gene products, growth factor receptors, transporters, and the like.

3.3. Lead Discovery and Development

Research applications notwithstanding, the ultimate value of the NCI in vitro screen will and should be judged by the extent to which it uniquely contributes to the discovery and development of new clinically useful anticancer drugs. As summarized elsewhere by Sausville (Chapter 11, this volume), from the tens of thousands of pure compounds tested initially in the in vitro primary screen, a subset was selected for in vivo preclinical followup. By the end of 1995, several of these compounds, which arguably were selected for NCI development based principally on their novel bioactivity profiles in the in vitro screen, were expected to achieve INDA approval by the FDA for phase 1 clinical trials. As also described separately by Plowman et al. (Chapter 6, this volume), continuing innovations and refinements of the in vivo preclinical followup evaluation of leads selected by the in vitro screen may both improve and accelerate discovery of the most novel and promising new leads for drug development.

4. CONCLUSION

It has been more than a decade since the concept for the NCI in vitro primary screen was first proposed. The initial 5 yr, 1985–1990, were consumed with developing key elements of the screening model, designing and constructing the physical facilities to accommodate the screening operations, recruiting and training staff, implementing and evaluating a pilot-scale screen and data management operations, and calibration and standardization of the screen. During the subsequent 5 yr of operation of the screen, 1990–1995, more than 100,000 materials, including pure compounds as well as natural product extracts, were tested either in the prescreen and/or the full screen assay against the 60-cell panel. The accrued data bases have provided a rich source of information that has proven to have considerable utility in certain research applications. Ongoing and future studies will likely reveal additional important research applications of the data accumulated to date. However, the prudence of continuing the 60-cell panel screening operations indefinitely into the future can appropriately be questioned. The decade of 1985–1995 was also one of exciting, if not explosive, progress in the understanding of the molecular gentics of cancer. This realization has been a stimulus to pursue the "molecular characterization" (*see* Sausville, Chapter 11, this volume) of the cell lines of the NCI screen with respect to selected genes, gene

products, and other possible "molecular targets" contributing to maintenance or reversion of the malignant phenotype. It is hoped that such information will facilitate use of the NCI 60-cell panel in vitro screen, and/or the accrued screening data bases therefrom, to discover novel molecular target-directed leads. Whether these and other potential research applications of the screen are sufficient to justify continuation is an appropriate matter for informed debate and consensus. Validation of any past, present, or future application of the NCI 60-cell screen as an effective tool for discovery of clinically useful new antitumor drugs, must await definitive clinical evaluation of new investigational agents whose discovery was uniquely dependent on the screen. Realistically that may require another decade into the future.

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APPENDICES

Appendix A. Participants [Non-NCI] in NCI Workshop on "Disease-Oriented Antitumor Drug Discovery and Development," January 9–10, 1985, Bethesda, MD

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Benjamin Winogard
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[a] Michael Alley subsequently joined the staff of DTP-NCI in 1987.
[b] Michael Grever subsequently joined the staff of DTP-NCI in 1990.

Appendix B
Members of Ad Hoc Review Committee for NCI In Vitro/In Vivo Disease-Oriented Screening Project

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Arthur Bogden
Michael Buas
Thomas Carey
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Thomas Corbett
Mortimer Elkind
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Heinz-Herbert Fiebig[c]
Øystein Fodstad
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Susan Horwitz
Peter Houghton
George Pettit[b]
Alexander Pihl
Alan Rosenthal
Sydney Salmon
Phillip Skehan[a]
Raymond Taetle
Peter Twentyman
Larry Weisenthal
Robert Whitehead

[a] Phillip Skehan served on the Committee prior to his joining the staff of DTP-NCI in 1987.
[b] John Faulkner, George Pettit and Sydney Hecht comprised an ad hoc advisory subcommittee in 1989 for the natural products program.
[c] Heinz-Herbert Fiebig served as a visiting scientist with DTP-NCI in 1988–89.
[d] Kenneth Harrap served as Chair of the Committee from its inception in 1985 through its last meeting in 1989.

Appendix C
Participants (Non-NCI) in NCI Workshop on "Selection, Characterization and Quality Controlof Human Tumor Cell-Linesfor the NCI's New Drug Screening Program," May 27–28, 1987, Bethesda, MD

Bruce Baguley	Steven Jacobs
Ralph Bernacki	Edward Keenan
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Arthur Bogden	Chung Lee
Michael Brattain	Al Liebovitz
Thomas Carey	John Masters
Desmond Carney	Stanley Mikulski
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[a] Phillip Skehan subsequently joined the staff of DTP-NCI (see footnote *a*, Appendix B).

ANTICANCER DRUG DEVELOPMENT GUIDE

PRECLINICAL SCREENING, CLINICAL TRIALS, AND APPROVAL

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6 Human Tumor Xenograft Models in NCI Drug Development

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CONTENTS

1. INTRODUCTION

The preclinical discovery and development of anticancer drugs by the NCI consist of a series of test procedures, data review, and decision steps that have been summarized recently (*1*). Test procedures are designed to provide comparative quantitative data, which in turn, permit selection of the best candidate agents from a given chemical or biological class. Periodic, comprehensive reviews by various NCI committees serve not only to identify and expedite the development of active lead compounds that may provide more efficacious treatments for human malignancy, but also to eliminate agents that are inactive and/or highly toxic from further consideration.

Various components in NCI's drug discovery and development process have evolved in response to a combination of factors—scientific, clinical, technological, and fiscal. A series of review articles have charted the evolution of the drug screening program and have described specific elements of the process, e.g., acquisition, screening, analog development and testing, pharmacology, and toxicology (*2–12*). The present chapter provides: a brief history of the in vivo screens used by NCI; a description of the human tumor xenograft systems, which are currently employed in preclinical drug development; a discussion of how these xenograft models are employed for both initial efficacy testing as well as detailed drug evaluations; and a description of a new model that may facilitate preclinical drug development.

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2. HISTORICAL DEVELOPMENT OF NCI SCREENS

Analyses of various screening methods available prior to 1955 indicated that (1) nontumor systems were incapable of replacing tumor systems as screens, and (2) no single tumor system was capable of detecting all active antitumor compounds (*13*). Since that time, the preclinical discovery and development of potentially useful anticancer agents by the NCI have utilized a variety of animal and human tumor models not only for initial screening, but also for subsequent studies designed to optimize antitumor activity of a lead compound or class of compounds. Although the various preclinical data review steps and criteria have remained essentially the same throughout the years, the modes and rationale of in vivo testing employed by NCI have evolved significantly.

2.1. Murine Tumor Screens, 1955–1975

In 1955, NCI initiated a large-scale in vivo anticancer drug screening program utilizing three murine tumor models: sarcoma 180, L1210 leukemia, and carcinoma 755. By 1960, in vivo drug screening was performed in L1210 and in two additional rodent models selected from a battery of 21 possible models. In 1965, screening was limited to the use of two rodent systems, L1210 and Walker 256 carcinosarcoma. In 1968, synthetic agents were screened in L1210 alone, whereas natural product testing was conducted in both L1210 and P388 leukemias. A special testing step was added to the screen in 1972 to evaluate active compounds against B16 melanoma and Lewis lung carcinoma. It is noteworthy that this first 20 years of in vivo screening relied heavily on testing conducted in the L1210 model.

2.2. Prescreen and Tumor Panel, 1976–1986

In late 1975, NCI initiated a new approach to drug discovery that involved prescreening of compounds in the ip-implanted murine P388 leukemia model, followed by evaluation of selected compounds in a panel of transplantable tumors (*14*). The tumors in the panel were chosen as representative of the major histologic types of cancer in the US and, for the first time in NCI history, included human solid tumors. The latter was made possible through the development of immunodeficient athymic (nu/nu) mice and transplantable human tumor xenografts in the early 1970s (*15,16*). Beginning in 1976, the tumor panel consisted of paired murine and human tumors of breast ($CD8F_1$ and MX-1), colon (colon 38 and CX-1 [the same as HT29]) and lung (Lewis and LX-1), together with the B16 melanoma and L1210 leukemia used in previous screens.

The majority of the early NCI testing conducted with the human tumors used small fragments growing under the renal capsule of athymic mice. The subrenal capsule (src) technique and assay were developed by Bogden and associates (*17*). Although labor-intensive, the src assay provided a rapid means of evaluating new agents against human tumor xenografts at a time when the testing of large numbers of compounds against sc xenografts seemed untenable. As experience was gained with the husbandry of athymic mice, longer-duration sc assays became manageable.

A detailed evaluation of the sensitivities of individual tumor systems employed from 1976–1982 revealed a wide range in sensitivity profiles as well as "yield" of active compounds (*14*). The data clearly indicated that rodent models may not be capable of detecting all compounds with potential activity against human malignan-

cies, and indicated that the best strategy for testing is to employ a combination of tumor systems to minimize loss of potentially useful compounds. These findings prompted the NCI in 1982 to develop a strategy for testing compounds that involved a sequential process of "progressive selection": NCI continued to use the P388 leukemia as a prescreen, but subsequent evaluation of selected agents was conducted in a modified tumor panel composed of "high-yield" models from the original panel (i.e., src-implanted MX-1 mammary carcinoma, and ip-implanted B16 melanoma and L1210 leukemia) and a new model, the ip-implanted M5076 sarcoma. Thereafter, evaluation of selected compounds would be "compound-oriented" and use protocols and models, selected on the basis of prior testing results and known properties of each compound, that would present the compound with increased biological and pharmacological challenge.

Alternate approaches to in vivo drug evaluation have been prompted by investigations on the metastatic heterogeneity of tumor cell populations. During the 1980s, several investigators associated with NCI conducted studies to assess the metastatic potential of selected murine and human tumor cell lines (B16, A-375, LOX-IMVI melanomas, and PC-3 prostate adenocarcinoma) and their suitability for experimental drug evaluation (e.g., *18–21*). A series of investigations by Fidler and associates demonstrated that metastasis is not random, but selective and that metastasis consists of a progression of sequential steps, the pattern of which is dependent on injection site (*22,23*). Such findings support the importance of establishing in vivo models derived from the implantation of tumor material in host tissues that are anatomically correct—"seed" and "soil" compatibility. Such "orthotopic" models also have been developed and utilized to study lung cancer (e.g., *24*), breast cancer (e.g., *25*), and prostate cancer (*26*). Although it may not be possible for NCI to employ these models in the initial steps of in vivo drug evaluations, such models may be well suited for subsequent, more detailed evaluation of compounds that exhibit activity against specific tumor types. Metastases and orthotopic models are discussed in greater detail in Chapters 7 and 8 of this volume.

2.3. Human Tumor Colony Formation Assay, 1981–1985

Based on initial reports by Salmon and colleagues (*27,28*), various clinical investigators working with fresh human tumor samples from patients and/or with early passage human tumor xenograft materials utilized various culture techniques to identify chemotherapeutic agents active against human malignancies (e.g., *29,30*). The NCI sponsored a pilot drug screening project utilizing a human tumor colony-forming assay (HTCFA) at multiple clinical cancer centers. Although it was possible to identify unique antitumor drug "leads" using such a technique, the HTCFA could be employed only for a limited number of tumor types and was not found suitable for large-scale drug screening (*31*).

2.4. Human Tumor Cell Line Screen, 1985–Present

In 1985, the NCI initiated a new project to assess the feasibility of employing human tumor cell lines for large-scale drug screening (*12; also see* Chapter 2 of this volume). Cell lines derived from seven cancer types (brain, colon, leukemia, lung, melanoma, ovarian, and renal) were acquired from a wide range of sources, cryopreserved, and subjected to a battery of in vitro and in vivo characterizations, including

testing in drug sensitivity assays. The approach was deemed suitable for large-scale drug screening in 1990 (*1*). With the implementation of a 60-member cell line in vitro screen, in vivo testing procedures were substantially altered as discussed below.

3. HUMAN TUMOR XENOGRAFT MODELS IN CURRENT USE

The new in vitro human tumor cell line screen shifted the NCI screening strategy from "compound-oriented" to "disease-oriented" drug discovery (*12*). Compounds of interest identified by the screen (e.g., those demonstrating disease-specific differential cytotoxicity) were to be considered "leads," requiring further preclinical evaluation to determine their therapeutic potential. As part of this followup testing, the antitumor efficacy of the compounds was to be evaluated in in vivo tumor models derived from the in vitro tumor lines used in the screen. Although only a subset of cell lines, selected on the basis of in vitro sensitivity, would be used for each agent, it was anticipated that for any selected compound, any cell line might be required as a xenograft model. In order to accomplish such an objective, a concerted developmental effort was required to establish a battery of human tumor xenograft models. As discussed below and elsewhere (*32*), tumorigenicity was demonstrated for the majority of the tumor lines utilized in the in vitro screen that became fully operational in April 1990 (*1*). Then, in 1993, composition of the cell line screen was modified: cell lines with variable growth characteristics and those providing redundant information were replaced by groups of prostate and breast tumor lines. As a consequence, additional xenograft model development was initiated for prostate and breast cancers.

3.1. Development of Human Tumor Xenografts

Efforts focused on the establishment of sc xenografts from human tumor cell-culture lines obtained from the NCI tumor repository at Frederick, MD. The approach is outlined in Fig. 1. The cryopreserved cell lines were thawed, cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone), and expanded until the population was sufficient to yield $\geq 10^8$ cells. Cells were harvested and then implanted sc into the axillary region of 10 athymic NCr nu/nu mice (1.0×10^7 cells/0.5 mL/mouse) obtained from the NCI animal program, Frederick, MD. Mice were housed in sterile, polycarbonate, filter-capped Microisolator™ cages (Lab. Products, Inc.), maintained in a barrier facility on 12-h light/dark cycles, and provided with sterilized food and water ad libitum. The implanted animals were observed twice weekly for tumor appearance. Growth of the solid tumors was monitored using *in situ* caliper measurements to determine tumor mass. Weights (mg) were calculated from measurements (mm) of two perpendicular dimensions (length and width) using the formula for a prolate ellipsoid and assuming a specific gravity of 1.0 g/cm^3– (*33*). Fragments of these tumors were subjected to histological, cytochemical, and ultrastructural examination to monitor the characteristics of the in vivo material and to compare them with those of the in vitro lines and, where possible, with those reported for initial patient tumors (*34*). Both in vitro and in vivo tumor materials exhibited characteristics consistent with tissue type and tumor of origin. However, not unexpectedly, differences in the degree of differentiation were noted between some of the cultured cell lines and corresponding xenograft materials.

The initial solid tumors established in mice were maintained by serial passage of 30–40 mg tumor fragments implanted sc near the axilla. There was an apparent cell

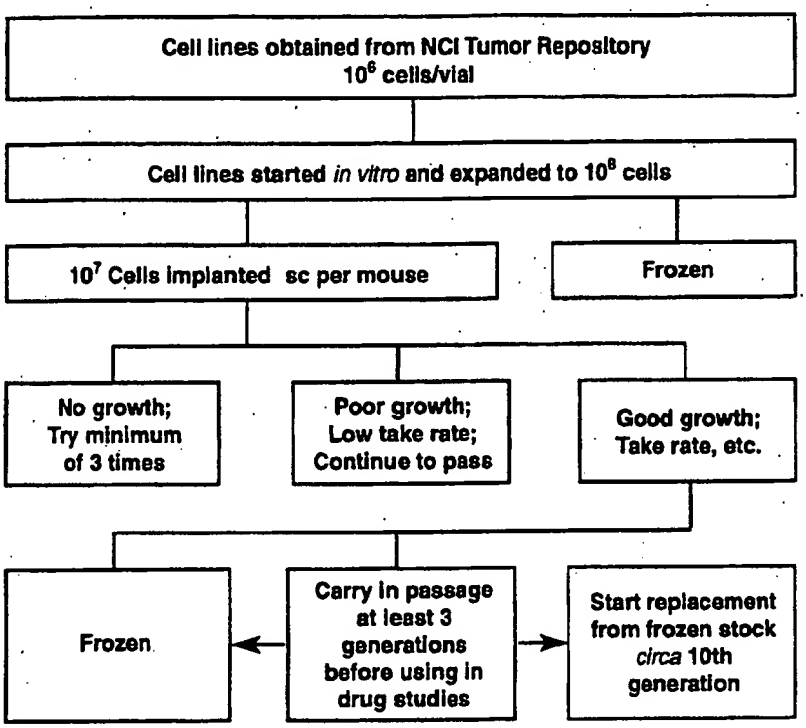

Fig. 1. Schematic of the development of in vivo models for drug evaluation.

population selection occurring in some of the tumors as they adapted to growth in animals during early in vivo passage, with growth rates increasing appreciably in sequential passages (*32*). Thus, xenografts were not utilized for drug evaluation until the volume-doubling time stabilized, usually around the fourth or fifth passage. The doubling time of xenografts derived from tumor cell lines constituting both the initial (1990) and the modified (1993) human tumor cell line screens, plus three additional breast tumors, are presented in Table 1. Also provided in the table is information on the take-rate of the tumors, and the experience of the NCI in the use of the tumors as early stage sc models. The doubling times were determined from vehicle-treated control mice used in drug evaluation experiments (only data for passage numbers 4–20 have been included). For each experiment, the doubling time is the median of the time interval for individual tumors to increase in size from 200–400 mg (usually a period of exponential growth). Both ranges and mean values are provided to demonstrate the inherent variability of growth for some of the xenograft materials even after a period of stabilization. Mean doubling times range from < 2 d for five tumors (SF-295 glioblastoma, MOLT 4 leukemia, DMS 273 small-cell lung tumor, and LOX-IMVI and SK-MEL-28 melanomas) to > 10 d for the MALME-3M and M19-MEL melanomas.

Difficulty was experienced in establishing and/or using some of the sc models. For example, even though HOP-62 nonsmall-cell lung tumors exhibited good growth rates, poor take-rates of 70, 50, 64, and 30% attained in the second through fifth passages, respectively, precluded their use for experimental drug testing. Although serial passage of OVCAR-3 ovarian tumors from sc-implanted fragments was difficult,

Table 1
Growth Characteristics of sc-Implanted Human Tumor Xenografts

Tumor Origin	Line	*In vitro* panel status 1990	*In vitro* panel status 1993	Mean volume doubling time (range) in days[a]	Take Rate[b]	Opinion for use as early-stage sc model
Colon	SW-620	Yes	Yes	2.4(1.7-3.9)	Good	Good
	KM12	Yes	Yes	2.4(1.9-3.3)	Good	Good
	HCT-116	Yes	Yes	2.6(1.8-3.4)	Good	Good
	HCT-15	Yes	Yes	3.4(1.8-5.0)	Good	Good
	HCC-2998	Yes	Yes	3.5(2.4-7.7)	Good	Acceptable
	DLD-1	Yes	No	3.8(3.1-5.5)	Good	Acceptable
	KM20L2	Yes	No	3.9(2.5-5.4)	Good	Acceptable
	COLO 205	Yes	Yes	4.3(2.4-8.9)	Good	Acceptable
	HT29	Yes	Yes	5.1(2.4-7.6)	Good	Acceptable
CNS	SF-295	Yes	Yes	1.4(1.0-2.0)	Good	Good
	SNB-75	Yes	Yes	3.1(2.0-4.6)	Good	Good
	U251	Yes	Yes	4.3(2.4-8.9)	Good	Good
	XF 498	Yes	No	4.4(2.6-8.3)	60-70%	Not Acceptable
	SNB-19	Yes	Yes	6.9(3.1-4.4)	60-70%	Not Acceptable
	SF-539	Yes	Yes	8.4(one only)	70%	Not Acceptable
	SF-268	Yes	Yes	NA[c]	Minimal growth	NA
	SNB-78	Yes	No	NA	No Growth	NA
Leukemia	MOLT-4	Yes	Yes	1.2(2.0-5.6)	80-100%	Acceptable
	HL-60(TB)[d]	Yes	Yes	3.3(2.1-4.9,ip)	85-100%(ip)	Good(ip)
	CCRF-CEM	Yes	Yes	4.6(4.3-4.6)	60-80%	Acceptable
	SR	Yes	Yes	5.1(one only)	80%	Not Acceptable
	RPMI-8226	Yes	Yes	NA	Minimal growth	NA
	K-562	Yes	Yes	NA	Minimal growth	NA
Lung: non-small cell	NCI-H460	Yes	Yes	2.1(1.3-3.0)	Good	Good
	NCI-H522	Yes	Yes	2.3(1.0-3.4)	Good	Good
	HOP-62	Yes	Yes	3.6(3.3-3.8)	30-65%	Not Acceptable
	NCI-H23	Yes	Yes	3.7(2.0-6.4)	Good	Good
	NCI-H322M	Yes	Yes	4.0(2.7-5.9)	Good	Acceptable
	EKVX	Yes	Yes	5.5(3.5-7.9)	Good	Acceptable
	HOP-92	Yes	Yes	6.0(5.1-8.4)	Good	Accepable
	A549/ATCC	Yes	Yes	8.4(5.8-10.9)	70-80%	Not Acceptable
	HOP-18	Yes	No	NA	Minimal growth	NA
	NCI-H266	Yes	Yes	NA	Minimal growth	NA

tumors grew more readily from sc implants of brei derived from ip-passaged material. The HL-60 (TB) promyelocytic leukemia did not grow well sc, but an ascitic ip line with a good take-rate was established successfully (Table 1). Growth characteristics of sc-implanted RXF 393 renal tumors are perhaps better suited for evaluation of a survival end point than for measurements of tumor size. Although demonstrating

Table 1 (Continued)

Tumor Origin	Line	*In vitro* panel status 1990	1993	Mean volume doubling time (range) in days[a]	Take Rate[b]	Opinion for use as early-stage sc model
Lung: small cell	DMS273	Yes	No	1.7(1.6-2.1)	Good	Good
	DMS114	Yes	No	4.8(2.8-7.5)	75-90%	Acceptable
Mammary	ZR-75-1	No	No	1.8(1.5-1.9)	Good	Good
	MX-1	No	No	2.7(2.2-3.0)	Good	Good
	UISO-BCA-1	No	No	4.1(2.8-4.8)	Good	Acceptable
	MDA MB-231/ATCC	No	Yes	4.4(2.7-7.7)	Good	Acceptable
	MCF7[e]	No	Yes	4.5(2.2-8.0)	Good	Acceptable
	MCF7/ADR-RES	No	Yes	6.1(4.2-7.9)	Good	Acceptable
	MDA-MB-435	No	Yes	6.6(2.8-13.6)	Good	Acceptable
	MDA-N	No	Yes	7.9(4.5-10.2)	Good	Acceptable
	HS578T	No	Yes	NA	Minimal growth	NA
	BT-549	No	Yes	NA	No growth	NA
	T-47D	No	Yes	NA	No growth	NA
Melanoma	LOX-IMVI	Yes	Yes	1.5(1.1-2.1)	Good	Good
	SK-MEL-28	Yes	Yes	1.9(1.1-2.5)	Good	Good
	UACC-62	Yes	Yes	2.8(1.8-4.2)	70-80%	Not Acceptable
	UACC-257	Yes	Yes	5.4(3.8-7.7)	Good	Acceptable
	SK-MEL-2	Yes	Yes	5.7(4.8-6.6)	80-90%	Not Acceptable
	M14	Yes	Yes	6.7(2.8-12.7)	Good	Acceptable
	SK-MEL-5	Yes	Yes	7.3(5.1-8.2)	Good	Acceptable
	MALME-3M	Yes	Yes	11.2(7.1-16.9)	80-90%	Not Acceptable
	M19-MEL	Yes	No	12.3(8.7-16.8)	60-90%	Not Acceptable
Ovarian	OVCAR-5	Yes	Yes	3.3(2.2-4.3)	Good	Good
	SK-OV-3	Yes	Yes	3.4(2.6-4.9)	Good	Good
	OVCAR-3[f]	Yes	Yes	5.5(5.0-5.9)	Good	Acceptable
	OVCAR-4	Yes	Yes	6.2(one only)	70-100%	Acceptable
	IGROV1	Yes	Yes	6.4(5.3-8.6)	Good	Acceptable
	OVCAR-8	Yes	Yes	12.2(11.2-13.0)	70%	Not Acceptable

(continued)

good initial growth, the RXF 393 tumors cause death in mice with low tumor burden, probably owing to paraneoplastic mechanisms. Other cell lines failed to become functional in vivo tumors, including two CNS, two nonsmall-cell lung, three breast, three renal tumor lines, and two leukemias, although minimal in vivo growth was observed with 9 of these 12 cultured lines (Table 1). With more extensive studies, it might be possible to attain improved tumor take-rates and growth by implanting tumors in

Table 1 (Continued)

Tumor Origin	Line	*In vitro* panel status 1990	*In vitro* panel status 1993	Mean volume doubling time (range) in days[a]	Take Rate[b]	Opinion for use as early-stage sc model
Prostate	PC-3	No	Yes	2.4(1.5-3.9)	Good	Good
	DU-145	No	Yes	4.4(2.0-7.9)	Good	Acceptable
Renal	CAKI-1	Yes	Yes	2.1(1.3-2.5)	Good	Good
	RXF 631	Yes	No	3.3(1.5-6.8)	Good	Acceptable
	A498	Yes	Yes	3.4(2.2-4.3)	Good	Acceptable
	RXF 393	Yes	Yes	3.4(2.3-5.7)	Good	Good
	SN12C	Yes	Yes	5.6(3.2-11.4)	Good	Acceptable
	786-0	Yes	Yes	6.7(one only)	80%	Not Acceptable
	ACHN	Yes	Yes	NA	Minimal growth	NA
	UO-31	Yes	Yes	NA	Minimal growth	NA
	TK-10	Yes	Yes	NA	No growth	NA

[a]Time for tumors to increase in size from 200–400 mg. Data are compiled from experiments using passage numbers 4–20. Tumors are listed in order of increasing mean doubling time/histologic type.
[b]Good: reproducible take-rate of ≥90%.
[c]NA: not applicable.
[d]Based on ip implant of 1.0×10^7 cells.
[e]MCF7 growth in athymic NCr nu/nu mice requires 17β-estradiol supplementation.
[f]Limited sc data obtained from implant of 0.5 mL 25% brei derived from ip-passaged tumor: poor growth is attained with serial passage of fragments from sc tumors.

severe combined immunodeficient (SCID) mice (scid/scid) (*35*). As discussed below, tumor take for some human lymphoma lines was markedly superior in SCID mice compared to athymic (nu/nu) (*36*) or triple-deficient BNX (bg/nu/xid) mice (*37*).

Establishment of breast tumor xenografts in vivo raised issues concerning hormonal requirements for growth of these tumors. For example, the importance of hormones in the growth of MCF7 breast carcinoma cells as solid tumors in athymic mice has been described (*38*). Our experience with this tumor also has shown the importance of 17 β-estradiol supplementation for the growth of the sc-implanted MCF7; 60-d release, 17 β-estradiol pellets (Innovative Research of America) are implanted sc in athymic mice 24 h prior to implanting MCF7 fragments in all NCI studies with this tumor. Growth of the remaining breast tumor xenografts appeared to be independent of estradiol supplements. For example, growth curves of individual early passage ZR-75-1 tumors implanted into athymic mice receiving either estradiol or no estradiol supplements completely overlapped (Fig. 2A), even though in this early passaged material, there was a large variation in the time postimplant for growth of individual tumors to be observed. The independence of ZR-75-1 tumor growth from estradiol supplements is further illustrated in Fig. 2B. Early stage vehicle-treated control tumors in mice receiving no estradiol supplementation demonstrate rapid growth (median doubling time 1.9 d) soon after implantation. The estrogen receptor (ER) status of the in vivo passaged ZR-75-1 tumors has not been determined, but the

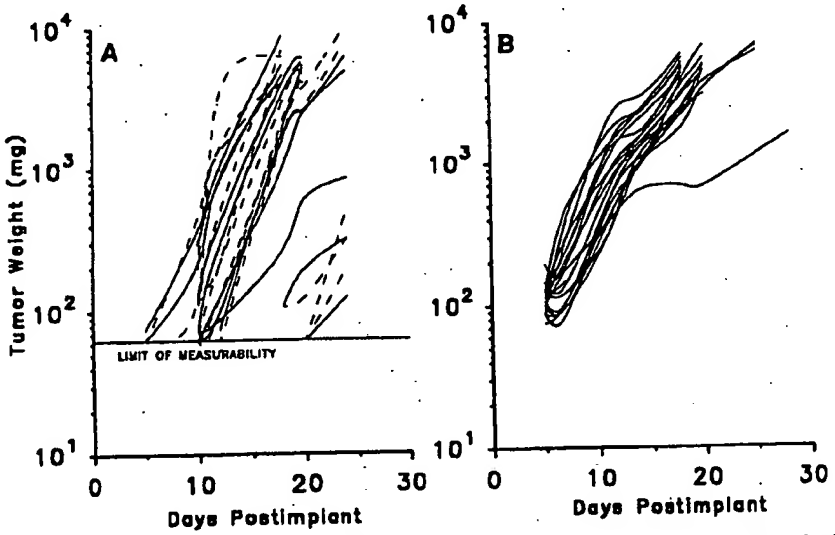

Fig. 2 Growth comparison of individual ZR-75-1 human breast tumor xenografts with and without estradiol supplementation. **A.** Passage number 5, – with 1.7 mg sc 17β-estradiol pellet implants; —without estradiol supplementation. **B.** Passage number 14, without estradiol supplementation.

growth characteristics of the tumors suggest an ER – status even though early evaluations of the in vitro cell line indicated an ER + status (*39*).

The in vivo growth characteristics of the xenografts determine their suitability for use in the evaluation of test agent antitumor activity, particularly when the xenografts are utilized as early stage sc models. For the purposes of the current discussion, the latter model is defined as one in which tumors are staged to 63–200 mg prior to the initiation of treatment. Our experience with the suitability of the xenografts as early stage models is listed in Table 1. Growth characteristics considered in rating tumors include take-rate, time to reach 200 mg, doubling time, and susceptibility to spontaneous regression. As can be noted, the faster-growing tumors tend to receive the higher ratings.

Since non-Hodgkin's lymphoma is one of the two principal malignancies occurring in the growing population of HIV-infected persons (*40*), the Developmental Therapeutics Program (DTP) has also established a group of human lymphoma xenografts for evaluating potential chemotherapeutic agents (*41*). This includes AS283, an Epstein-Barr virus (EBV)-positive, HIV-negative Burkitt's lymphoma derived from an AIDS patient (*42*); KD488, an EBV-negative, pediatric Burkitt's lymphoma (*43–46*); and RL, a diffuse, small noncleaved B-cell lymphoma (*47*). These lines grow sc with take-rates in excess of 90% in SCID mice, whereas much lower take-rates occur in athymic or triple-deficient BNX mice. Our finding of greater take-rates for the human leukemias/lymphomas in SCID mice compared to athymic mice is consistent with the known capacity of SCID mice to support xenografts of normal human hematopoietic cells (*48*).

3.2. Advanced-Stage sc Xenograft Models

Advanced-stage sc-implanted tumor xenograft models were established originally for use in evaluating the antitumor activity of test agents, so that clinically relevant

parameters of activity could be determined, i.e., partial and complete regressions, durations of remission (*49–51*). Tumor growth is monitored, and test agent treatment is initiated when tumors reach a weight range of 100–400 mg (staging day, median weights approx 200 mg), although depending on the xenograft, tumors may be staged at larger sizes. Tumor size and body weights are obtained approximately 2 times/wk and entered into DTP's DEC 10,000 Model 720 AXP computer. Through software programs developed by staff of the Information Technology Branch of DTP, in particular by David Segal and Penny Svetlik, data are stored, various parameters of effect are calculated, and data are presented in both graphic and tabular formats. Parameters of toxicity and antitumor activity are defined as follows:

1. Parameters of toxicity: Both drug-related deaths (DRDs) and maximum percent relative mean net body weight losses are determined. A treated animal's death is presumed to be treatment-related if the animal dies within 15 d of the last treatment, and either its tumor weight is less than the lethal burden in the control mice, or its net body weight loss at death is 20% greater than the mean net weight change of the controls at death or sacrifice. A DRD also may be designated by the investigator. The mean net body weight of each group of mice on each observation day is compared to the mean net body weight on staging day. Any weight loss that occurs is calculated as a percent of the staging day weight. These calculations also are made for the control mice, since tumor growth of some xenografts has an adverse effect on the weight of the mice.
2. Optimal % T/C: Changes in tumor weight (Δ weights) for each treated (T) and control (C) group are calculated for each day tumors are measured by subtracting the median tumor weight on the day of first treatment (staging day) from the median tumor weight on the specified observation day. These values are used to calculate a percent T/C as follows:

$$\%\ T/C = (\Delta T / \Delta C) \times 100 \text{ where } \Delta T > 0 \text{ or}$$
$$= (\Delta T / T_I) X\ 100 \text{ where } \Delta T < 0 \quad (1)$$

   and T_I is the median tumor weight at the start of treatment. The optimum (minimum) value obtained after the end of the first course of treatment is used to quantitate antitumor activity.
3. Tumor growth delay: This is expressed as a percentage by which the treated group weight is delayed in attaining a specified number of doublings (from its staging day weight) compared to controls using the formula:

$$[(T - C) / C] \times 100 \quad (2)$$

   where T and C are the median times in days for treated and control groups, respectively, to attain the specified size (excluding tumor-free mice and DRDs). The growth delay is expressed as percentage of control to take into account the growth rate of the tumor since a growth delay based on T − C alone varies in significance with differences in tumor growth rates.
4. Net log cell kill: An estimate of the number of $\log_{10}$ units of cells killed at the end of treatment is calculated as:

$$\{[(T - C) - \text{duration of treatment}] \times 0.301 / \text{median doubling time}\} \quad (3)$$

   where the doubling time is the time required for tumors to increase in size from 200–400 mg, 0.301 is the $\log_{10}$ of 2, and T and C are the median times in days for treated and control tumors to achieve the specified number of doublings. If the duration of treat-

ment is 0, then it can be seen from the formulae for net log cell kill and percent growth delay that log cell kill is proportional to percent growth delay. A log cell kill of 0 indicates that the cell population at the end of treatment is the same as it was at the start of treatment. A log cell kill + 6 indicates a 99.9999% reduction in the cell population.

5. Tumor regression: The importance of tumor regression in animal models as an end point of clinical relevance has been propounded by several investigators (*49–51*). Regressions are defined as partial if the tumor weight decreases to 50% or less of the tumor weight at the start of treatment without dropping below 63 mg (5 *X* 5 mm tumor). Both complete regressions (CRs) and tumor-free survivors are defined by instances in which the tumor burden falls below measurable limits (< 63 mg) during the experimental period. The two parameters differ by the observation of either tumor regrowth (CR) or no regrowth (tumor-free) prior to the final observation day. Although one can measure smaller tumors, the accuracy of measuring a sc tumor smaller than 4 × 4 or 5 × 5 mm (32 and 63 mg, respectively) is questionable. Also, once a relatively large tumor has regressed to 63 mg, the composition of the remaining mass may be only fibrous material/scar tissue. Measurement of tumor regrowth following cessation of treatment provides a more reliable indication of whether or not tumor cells survived treatment.

Most xenografts that grow sc are amenable to use as an advanced-stage model, although for some tumors, the duration of the study may be limited by tumor necrosis. As mentioned previously, this model enables the investigator to measure clinically relevant parameters of antitumor activity and provides a wealth of data on the effects of the test agent on tumor growth. Also, by staging day, the investigator is ensured that angiogenesis has occurred in the area of the tumor, and staging enables no-takes to be eliminated from the experiment. However, the model can be costly in terms of time and mice. For the slower-growing tumors, the passage time required before sufficient mice can be implanted with tumors may be at least 3–4 wk, and an additional 2–3 wk may be required before the tumors can be staged. In order to stage tumors, more mice than needed for actual drug testing must be implanted, often 50%, and sometimes 100% more.

3.3. Early Treatment and Early Stage sc Xenograft Models

Early treatment and early stage sc models are similar to the advanced-stage model, but because treatment is initiated earlier in the development of the tumor, the models are not suitable for tumors that have less than a 90% take-rate or have a > 10% spontaneous regression rate. We define the early treatment model as one in which treatment is initiated before tumors are measurable, i.e., < 63 mg, and the early stage model as one in which treatment is initiated when tumor size ranges from 63–200 mg. The 63-mg size is used as an indication that the original implant of approx 30 mg has demonstrated some growth. Parameters of toxicity are the same as those for the advanced-stage model; parameters of antitumor activity are similar. Percent T/C values are calculated directly from the median tumor weights on each observation day instead of as changes (Δ) in tumor weights, and growth delays are based on the time in days after implant for the tumors to reach a specified size, e.g., 500 or 1000 mg. Tumor-free mice are recorded, but may be designated no-takes or spontaneous regressions if the vehicle-treated control group contains more than 10% mice with similar growth characteristics. A no-take is a tumor that fails to become established and grow progressively. A spontaneous regression (graft failure) is a tumor that, after

a period of growth, decreases to 50% or less of its maximum size. Tumor regressions are not normally recorded, since they are not always a good indicator of antineoplastic effects in the early stage model. For those experiments in which treatment is initiated when tumors are 100 mg or less, only a minimal reduction in tumor size may bring the tumor below the measurable limit, and for some small tumors early in their growth, reductions in tumor size may reflect erractic growth rather than a true reflection of a cell killing effect. The big advantage of the early treatment model is the ability to use all implanted mice. The latter is the reason for requiring a good tumor take-rate, and in practice, the tumors most suitable for this model tend to be the faster-growing ones.

3.4. Challenge Survival Models

Although not utilized to a significant degree in the current NCI program, a few studies are conducted that depend on determining the effect of human tumor growth on the life-span of the host. Three tumors have been used as ip-implanted models: the HL-60 (TB) promyelocytic leukemia, the LOX-IMVI melanoma, and the OVCAR-3 ovarian carcinoma. Also, the SF-295 and U251 glioblastomas have been implanted intracerebrally. All mice dying or sacrificed owing to a moribund state or extensive ascites prior to the final observation day are used to calculate median days of death for treated (T) and control (C) groups. These values are then used to calculate a percent increase in life-span as follows:

$$\%\ \text{ILS} = [(T - C)/C] \times 100 \quad (4)$$

Wherever possible, titration groups are included to establish a tumor doubling time for use in $\log_{10}$ cell kill calculations. Laboratory personnel may designate a death (or sacrifice) as drug-related based on visual observations and/or the results of necropsy. Otherwise, treated animal deaths are designated as treatment-related if the day of death precedes the mean day of death of the controls minus 2 SD or if the animal dies without evidence of tumor within 15 d of the last treatment.

3.5. Response of Xenograft Models to Standard Agents

The drug sensitivity profiles for the advanced-stage sc xenograft models in our program have been established using 12 clinical antitumor drugs (Table 2). Each of these agents, obtained from the Drug Synthesis and Chemistry Branch, DTP, were evaluated following ip administration at multiple dose levels. The activity ratings are based on the optimal effects attained with the maximally tolerated dose ($< LD_{10}$) of each drug for the treatment schedule shown. The latter were selected on the basis of the doubling time of a given tumor, with longer intervals between treatments for slower-growing tumors. Apparent inconsistencies between the doubling times shown in Table 1 and selected schedules in Table 2 are the result of increased tumor growth rates for some tumors in the later studies depicted in Table 1. In later chemotherapeutic trials with breast tumors, paclitaxel was included in the group of clinical drugs evaluated, and drug characteristics were considered to some extent in the selection of treatment regimens (Table 3).

With the caveat that no attempts were made to optimize drug administration in each model, it can be seen that at least minimal antitumor effects (%T/C $\leq$ 40) were

Table 2
Response of Staged sc-Implanted Human Tumor Xenografts to 12 Clinical Anticancer Drugs[b]

Tumor	IP Treatment Schedule	Alkylating Agents						DNA Binders			Antimetabolites		Mitotic Inhibitor
		L-PAM	CYT	DTIC	BCNU	MMC	DDPt	Act D	ADR	BLEO	MTX	5FU	VBL
Colon:													
SW-620	q7dx3	1	0	4	3	4	1	0	0	1	0	0	0
KM12	q4dx3	NA	NA	0	1	NA	0	0	NA	1	0	0	0
HCT-116	q4dx3	NA	0	0	0	1	0	1	NA	1	0	0	0
HCT-15	q7dx3	1	0	0	0	1	0	1	0	0	0	0	0
HCC-2998	q4dx3	0	0	0	0	4	0	0	0	1	0	0	0
KM20L2	q4dx3	0	1	0	0	1	1	0	0	0	0	1	0
COLO 320DM	q4dx3	0	0	3	0	0	NA	0	0	0	0	1	0
COLO 205	q4dx3	1	0	0	0	3	1	1	0	0	0	1	1
HT29	q4dx3	0	0	0	0	2	2	0	0	1	0	0	0
CNS:													
SF-295	q4dx3	0	1	0	1	0	1	0	0	0	0	0	4
U251	q4dx3	0	2	4	4	3	3	1	0	0	0	0	1
XF 498	q7dx3	0	0	4	3	1	1	0	1	0	0	0	0
SNB-19	q4dx3	1	2	3	NA	3	NA	0	NA	NA	0	0	1
Leukemia:													
MOLT-4	q4dx3	4	1	0	0	1	1	0	1	1	1	0	0
Lung, non-small cell:													
NCI-H460	q4dx3	NA	0	NA	NA	4	NA	0	0	1	NA	0	0
NCI-H522	q4dx3	1	0	0	NA	≥2	1	NA	1	NA	NA	0	NA
HOP-62	q4dx3	2	NA	NA	NA	1	1	0	0	1	0	0	1
Lung, non-small cell:													
NCI-H23	q4dx3	3	1	0	0	4	4	1	1	1	1	1	0
NCI-H322M	q7dx3	0	0	0	0	4	1	0	0	0	0	1	0
EKVX	q4dx3	1	1	1	0	1	0	0	0	1	0	0	1
HOP-92	q4dx3	1	0	4	2	0	1	0	1	0	0	0	1
Lung, small cell:													
DMS 273	qdx4	1	1	0	0	1	0	1	0	1	1	1	2
DMS 114	q4dx3	0	1	0	1	1	0	0	0	0	0	0	1
NCI-H69	q4dx3	4	1	3	4	2	NA	0	NA	0	0	1	0
Melanoma:													
LOX-IMVI	qdx5	1	2	2	2	2	1	0	NA	0	NA	0	2
SK-MEL-28	q4dx3	0	1	0	0	1	0	0	0	0	0	0	0
UACC-62	q7dx3	0	0	1	1	1	1	1	1	1	0	0	1
SK-MEL-31	q4dx3	0	0	0	NA	1	1	1	0	NA	0	0	NA
UACC-257	q7dx3	0	0	4	1	2	1	1	1	0	0	1	0
SK-MEL-2	q7dx3	1	0	0	0	1	1	1	0	1	0	1	2
M14	q4dx3	0	0	0	0	0	0	0	1	1	0	1	0
MALME-3M	q4dx3	1	0	4	1	1	1	1	0	1	0	0	0
Ovarian:													
SK-OV-3	q7dx3	0	0	NA	NA	1	0	0	0	1	0	0	0
IGROV1	q4dx3	1	0	0	0	1	1	1	0	1	1	1	0
OVCAR-8	q7dx3	0	0	0	0	1	1	0	1	0	0	0	0

(continued)

produced in each tumor model by at least 2, and as many as 10, clinical drugs (Table 4). The number of responses appeared to be independent of doubling time and histological type with a range in the number of responses observed for tumors in each subpanel. When the responses are considered in terms of the more clinically relevant end points of partial or complete tumor regression, it can be seen that the tumor models were quite refractory to standard drug therapy with 30 of 48 (62.5%) not responding

Table 2 (Continued)

Tumor	IP Treatment Schedule	Alkylating Agents						DNA Binders			Antimetabolites		Mitotic Inhibitor
		L-PAM	CYT	DTIC	BCNU	MMC	DDPt	Act D	ADR	BLEO	MTX	5FU	VBL
Prostate:													
PC-3	qdx4	1	0	1	0	0	0	0	0	1	1	0	0
DU-145	q4dx3	NA	1	NA	NA	NA	1	0	NA	NA	1	0	0
Renal:													
CAKI-1	q7dx3	1	1	0	0	1	1	1	1	0	0	1	0
SN12K1	q7dx3	0	0	0	0	4	0	0	0	0	0	0	1
A498	q7dx3	0	0	1	1	1	1	0	1	1	0	0	0
RXF 393	q4dx3	1	1	1	1	2	1	0	1	1	0	0	1
SN12C	q7dx3	0	0	0	1	0	0	0	0	1	0	0	0
786-0	q7dx3	0	NA	NA	NA	1	1	0	NA	NA	0	NA	NA

[a] Standard agents are melphalan (L-PAM), cytoxan (CYT), dacarbazine (DTIC), 1, 3-bis (2-chloroethyl)-1-nitrosourea (BCNU), mitomycin C (MMC), cisplatin (DDPt), actinomycin D (act D), doxorubicin (ADR), bleomycin (BLEO), methotrexate (MTX), 5-fluorouracil (5FU), vinblastine (VBL).

[b] Activity rating based on optimal %Δ T/Δ C attained after treatment had ended:
0 = Inactive, %T/C > 40.
1 = Tumor inhibition, %T/C range 1–40.
2 = Tumor stasis, %T/C range 0 to −49.
3 = Tumor regression, %T/C range −50 to −100.
4 = %T/C range −50 to −100 and >30% tumor-free mice at experiment end.

Table 3
Response of Staged sc-Implanted Breast Tumor Xenografts to 13 Clinical Anticancer Drugs[a]

Tumor[b]	Alkylating Agents						DNA Binders			Antimetabolites		Mitotic Inhibitor	
	L-PAM	CYT	DTIC	BCNU	MMC	DDPt	Act D	ADR	BLEO	MTX	5-FU	VBL	PAC
ZR-75-1	1[b]	1	4	1	1	1	1	3	1	0	0	1	NA
MX-1	4	4	0	1	4	4	0	2	1	1	0	0	4
UISO-BCA-1	0	0	1	0	NA	0	0	0	0	NA	0	0	3
MCF7	1	0	1	1	1	0	0	0	1	0	NA	0	0
MDA-MB-435	1	0	4	3	NA	0	NA	0	1	NA	0	1	4
MDA-N	0	0	4	0	0	0	1	1	0	0	NA	0	3

[a] Clinical drugs include those listed in Table 2, plus paclitaxel (PAC). Treatment regimens were ip qd x 5 for MTX and 5FU, ip q4d x 3 for L-PAM, CYT, DTIC, BCNU, MMC, DDPt, Act D, BLEO, and VBL, iv q4d x 3 for ADR, and iv qd x 5 for PAC.

[b] Activity rating: *see* legend to Table 2.

to any of the drugs tested (Table 4). As tested, the clinical drugs producing the highest response rates (number of tumors responding [%T/C ≤ −50%]/ total tumors evaluated) were DTIC (11/44) and mitomycin C (9/45) (Tables 2 and 3). Paclitaxel was not evaluated in the majority of tumors, but demonstrated excellent activity in four of five breast tumor models (Table 3).

Table 4
Response of Staged sc Human Tumor Xenografts to Clinical Anticancer Drugs

Panel	Tumor	Number of drugs active[a]: Minimal activity[b]	Tumor regression[c]
Colon	SW-620	6	3
	KM12	2/8	0/8
	HCT-116	3/10	0/10
	HCT-15	3	0
	HCC-2998	2	1
	KM20L2	4	0
	COLO 320DM	2/11	0/11
	COLO 205	6	1
	HT29	3	0
CNS	SF-295	4	1
	U251	7	4
	XF 498	5	2
	SNB-19	5/8	2/8
Lung, non-small cell	NCI-H460	2/7	1/7
	NCI-H522	4/7	0/7
	HOP-62	5/9	0/9
	NCI-H23	9	3
	NCI-H322M	3	1
	EKVX	6	0
	HOP-92	6	1
Lung, small cell	DMS 273	8	0
	DMS 114	4	0
	NCI-H69	6/10	3/10
Mammary	ZR-75-1	10/12	2/12
	MX-1	9	5
	UISO-BCA-1	2/11	1/11
	MCF7	5/12	0/12
	MDA-MB-435	6/10	3/10
	MDA-N	4/12	2/12

(continued)

3.6. Strategy for Initial Compound Evaluation In Vivo

The in vitro primary screen provides the basis for selection of the most appropriate lines to use for the initial followup in vivo testing, with each compound tested only against xenografts derived from cell lines demonstrating the greatest sensitivity to the

Table 4 (Continued)

Panel	Tumor	Number of drugs active[a]	
		Minimal activity[b]	Tumor regression[c]
Melanoma	LOX-IMVI	7/10	0
	SK-MEL-28	2	0
	UACC-62	8	0
	SK-MEL-31	3/9	0/9
	UACC-257	7	0
	SK-MEL-2	7	0
	M14	3	0
	MALME-3M	7	1
Ovarian	SK-OV-3	2/10	0/10
	IGROV1	7	0
	OVCAR-8	3	0
Prostate	PC-3	4	0
	DU-145	3/6	0/6
Renal	CAKI-1	7	0
	SN12K1	2	0
	A498	6	0
	RXF 393	9	0
	SN12C	2	0
	786-0	2/5	0/5

[a] Except where noted, the number of clinical drugs evaluated was 12 for the tumors listed in Table 2, and 13 for the breast tumors listed in Table 3.
[b] % T/C ≤ 40, ratings 1–4 in Tables 2 and 3.
[c] % T/C ≤ −50, ratings 3 and 4 in Tables 2 and 3.

agent in vitro. Our early strategy for in vivo testing emphasized the treatment of animals bearing advanced-stage tumors. Examples of the in vivo data obtained with one such agent are summarized in Table 5. The quinocarmycin derivative DX-52-1, identified as a melanoma-specific agent in vitro, demonstrated statistically significant antitumor activity against five of seven melanoma xenografts following ip administration on intermittent schedules (*52*). The best in vivo activity was observed against the rapidly dividing LOX-IMVI melanoma.

The strategy for in vivo testing has undergone some modifications as experience has been gained with the screen and the xenograft models. Currently, dose range finding studies in nontumored mice are conducted for new compounds identified by the in vitro screen. Unless information is available to guide dose selection, single mice are treated with single ip bolus doses of 400, 200, and 100 mg/kg and observed for 14 d. Sequential three-dose studies are conducted as necessary, until a nonlethal dose range is established, after which the compound is evaluated in the ip-implanted murine P388 leukemia model on an ip multidose treatment regimen. The latter provides dos-

Table 5
Response of Advanced-Staged sc Human Melanoma Xenografts to the Quinocarmycin Derivative, DX-52-1[a]

Melanoma	Treatment days	Dose (ip, mg/kg/day)[b]	Optimal %T/C (Day)[c]	Growth delay: %(T-C)/C[d]	Regressions Complete	Regressions Partial
LOX-IMVI	5, 9, 13	90	-54	181	2/10	3/10
	5, 9, 13, 17 21, 25	60	-100*	389	4/6	0/6
SK-MEL-2	14, 21, 28	90	10(32)	118	2/6	0/6
SK-MEL-5	15, 19, 23	40	49(33)	27	0/6	0/6
UACC-62	16, 23, 30	90	18(34)	185	0/7	0/7
UACC-257	16, 20, 24	90	12(27)	35	0/6	0/6
M14	12, 16, 20	90	19(26)	56	0/6	0/6
MALME-3M	27, 31, 35	90	64(72)	4	0/6	0/6

[a] Adapted from ref. *52*.
[b] Maximally tolerated dose, $\leq LD_{10}$ and < 20% net body wt loss.
[c] *See* Section 3.2., Step 2 for calculation of % T/C. The number in parenthesis is the day on which the optimal (minimum) T/C was attained.
[d] *See* Section 3.2., Step 3 for calculation of growth delay. Based on an end point of two doublings (four for LOX-IMVI).

ing information for the more costly xenograft models and data for retrospective comparison with previous NCI screening. The test agent is then evaluated in three sc xenograft models using tumors that were among the most sensitive to the test agent in vitro and that are suitable for use as early staged models (Table 1). The compounds are administered ip, often as suspensions, on schedules based, with some exceptions, on the mass doubling time of the tumor. For doubling times of 1.3–2.5, 2.6–5.9, and 6–10 d, the schedules are daily for five treatments (qd × 5), every fourth day for three treatments (q4d × 3), and every seventh day for three treatments (q7d × 3). For most tumors, the interval between individual treatments approximates the doubling time of the tumors, and the treatment period allows a 0.5–1.0 $\log_{10}$ unit of control tumor growth. For tumors staged at 100–200 mg, the tumor sizes of the controls at the end of treatment range from 500–2000 mg, which allows sufficient time after treatment to evaluate the effects of the test agent before it becomes necessary to sacrifice mice owing to tumor size.

Table 6
Effect of Route of Administration on the Activity of Paclitaxel Against Staged sc-Implanted MX-1 Mammary Carcinoma Xenografts

Treatment		Complete	Tumor-	Minimum	Growth	Net Log
Route, Schedule	Opt. Dose (mg/kg/day)[a]	Regressions /Total	Free on Day 40	%T/C[b] (Day)	Delay: %(T-C)/C[c]	Cell Kill
iv, D8-12	22.5	1/9	8	-100(15)[d]	449	2.9
ip, D8-12	15.0	2/9	1	28(19)	29	-0.2
iv, D8,12,16	22.5	6/9	2	-100(15)	357	2.1
ip, D8,12,16	30	0/9	0	20(22)	70	-0.3

[a]Paclitaxel was administered as a solution in 12.5% ethanol: 12.5% cremophor: 75% normal saline at multiple dose levels. Data from doses ($\leq LD_{10}$) producing the optimal effects are shown.
[b]*See* Section 3.2. for an explanation of the parameters of antitumor effects.
[c]Based on an end point of two doublings, C = 7.1 d. Tumor-free mice and mice dying of apparent drug-related effects were excluded from the calculations.
[d]Number in parentheses indicates the observation day on which data were obtained.

3.7. Detailed Drug Studies

Once a compound has been identified that demonstrates some in vivo efficacy in initial evaluations, more detailed studies can be designed and conducted in human tumor xenograft models to explore further the compound's therapeutic potential. By varying the concentration and exposure time of the tumor cells and the host to the drug, it is possible to devise and recommend treatment strategies designed to optimize antitumor activity. As many of the initial in vivo studies deliver suspensions of the compound into the peritoneal cavity, it is unlikely that the sc tumors receive optimal concentrations of, and exposure to, test agents. The early preclinical antitumor evaluation of paclitaxel illustrates this problem. In NCI studies, prior to its clinical evaluation, paclitaxel had demonstrated its best effects against ip-implanted tumors, and no activity was observed in sc models following ip administration as a suspension (*53, 54*). Later investigations demonstrated that sc-implanted MX-1 mammary carcinoma xenografts were highly responsive to treatment with iv solutions of paclitaxel (*55*), although they had failed to respond to treatment with ip suspensions (*54*). As illustrated in Table 6, iv solutions of paclitaxel administered on either a daily or intermittent schedule produced complete tumor regressions in the majority of the treated mice. Some of these mice remained tumor-free 24–28 d after the last treatment, and tumor growth delays in the remaining mice were excellent. In contrast, only modest antitumor effects were observed following the ip administration of paclitaxel solutions. Pharmacokinetic data obtained in mice indicated only 10% bioavailability of paclitaxel from ip administration (*56*).

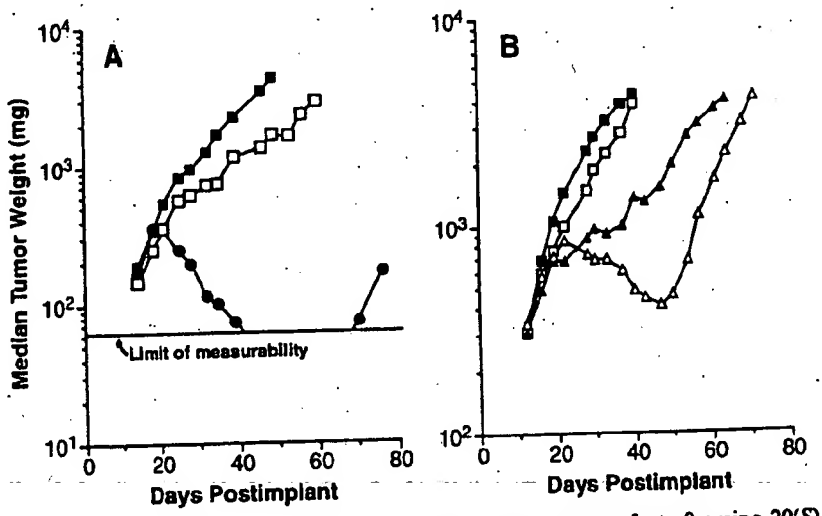

Fig. 3 Response of advanced-stage sc human HT29 colon tumor xenografts to 9-amino-20(*S*)-camptothecin (9-AC). A. ■—■, Vehicle-treated (saline:tween 80) controls; ●—●, 4 mg/kg/d administered as an sc bolus suspension in saline:tween 80; □—□, 4 mg/kg/d administered as an sc bolus solution in propylene glycol (PG):polyethylene glycol 400 (PEG 400): DMSO (95% of an 80% PG: 20% PEG mixture + 5% DMSO). Treatments were administered on a q4d x 8 schedule starting 14 d postimplant. B. ■—■, Vehicle-treated (saline:tween 80) controls; □—□, 4 mg/kg/d sc q4d x 8; ▲—▲, 3 mg/kg/d, sc q2d x 16; Δ—Δ, 1 mg/kg/d, sc qd x 32. All 9-AC treatments were given as solutions in the PG:PEG:DMSO vehicle starting on day 12.

The importance of "concentration x time" on the antitumor effects of test agents is well illustrated by data obtained with 9-amino-20(*S*)-camptothecin (9-AC). Although an sc bolus injection of a 9-AC suspension caused complete tumor regression of advanced-stage sc-implanted human HT29 colon tumor xenografts, only a marginal growth-inhibitory effect was observed when 9-AC was administered as a solution (Fig. 3A). Augmented activity with the solution was obtained by increasing the frequency of administration (Fig. 3B). Characterization of the plasma pharmacokinetics in mice, conducted in conjunction with the efficacy studies, indicated that absorption of 9-AC from sc-injected solutions was rapid and efficient, but elimination also was fast (*57*). The plasma profile afforded from the suspension differed profoundly: peak plasma levels were lower and the rate of elimination much slower (*57*). The studies indicated that maintaining the 9-AC lactone plasma concentration above a threshold level for a prolonged period of time was required for optimal therapeutic effects.

4. HOLLOW-FIBER ASSAYS: A NEW APPROACH TO IN VIVO DRUG TESTING

DTP is evaluating the suitability of a model that would be integrated into the present drug development scheme between the in vitro 60 cell line screen and the in vivo sc xenograft assays. This model, based on human tumor cell lines growing in hollow fibers, is being developed as a prioritization tool through which lead compounds

identified in the in vitro screen would pass (*58*). The goal is to direct the most promising compounds into in vivo testing as rapidly as possible after their selection for testing from the in vitro screening data. Presently, 10,000 compounds are screened in vitro each year and 8–10% of these are referred for in vivo testing. Current resources provide testing for approx 300–350 compounds in the xenograft models annually. Thus, the in vivo evaluation of compounds for activity is limited by the availability of testing resources. A method for prioritizing compounds for testing in the xenograft models, and for identifying the most sensitive cell lines to use in the models, would increase the prospect of rapidly identifying those compounds with the greatest potential for having in vivo efficacy. The hollow-fiber assay is intended to serve this purpose. In brief, tumor cells are inoculated into hollow fibers (1-mm internal diameter), and the fibers are heat-sealed and cut at 2-cm intervals. These samples are cultivated for 24–48 h in vitro and then implanted into athymic (nu/nu) mice. At the time of implantation, a representative set of fibers is assayed for viable cell mass by the "stable-end point" MTT dye conversion technique (*59*) in order to determine the time zero cell mass for each cell line. The mice are treated with experimental therapeutics on a daily treatment schedule, and the fibers are collected 6–8 d postimplantation. At collection, the quantity of viable cells contained in the fibers is measured. The antitumor effects of the test agent are determined from the changes in viable cell mass in the fibers collected from compound-treated and diluent-treated mice. Using this technique, three different tumor cell lines can be grown conveniently in each of two physiologic sites (ip and sc) within each experimental mouse (*58*). Thus, this model provides a method whereby a test agent can be administered ip to evaluate its effect against tumor cells growing in both the ip cavity and the sc compartment. With this simultaneous assessment of multiple tumor cell lines grown in two physiologic compartments, it is possible to identify lead compounds rapidly with the greatest promise of in vivo activity.

A graphic representation of typical data generated with this assay is shown in Fig. 4. The experimental mice received an ip and an sc hollow fiber of each of three human cell lines (COLO 205 colon, U251 CNS, and OVCAR-5 ovarian tumors) being evaluated. The mice were treated ip with the test agent on days 1–4, and the hollow fibers were removed on day 6. The viable cell mass was determined for the fiber samples, and the percent net growth of each cell line was calculated with reference to the time zero viable cell mass present in the hollow fibers on the day of implantation into mice. The net growth of each cell line in the ip and sc fibers is shown in Fig. 4A and B, respectively. The test agent was effective in suppressing growth of all three cell lines with the greater activity measured against ip implants. Of the cell lines tested, COLO 205 was the most sensitive, a finding that was confirmed by the results of the sc xenograft evaluations conducted following the hollow-fiber assay.

Experimentation to date indicates that this in vivo/in vitro hollow-fiber system may be well suited for the prioritization of compounds for more advanced stages of in vivo drug evaluation. In a practical sense, this hollow-fiber system is viewed as a means to facilitate traditional chemotherapeutic testing, since it is rapid, appears to be sensitive, and is broadly applicable to a variety of human tumor cell types. Additionally, it requires only a limited quantity of test compound, a small number of animals, and very limited animal housing space.

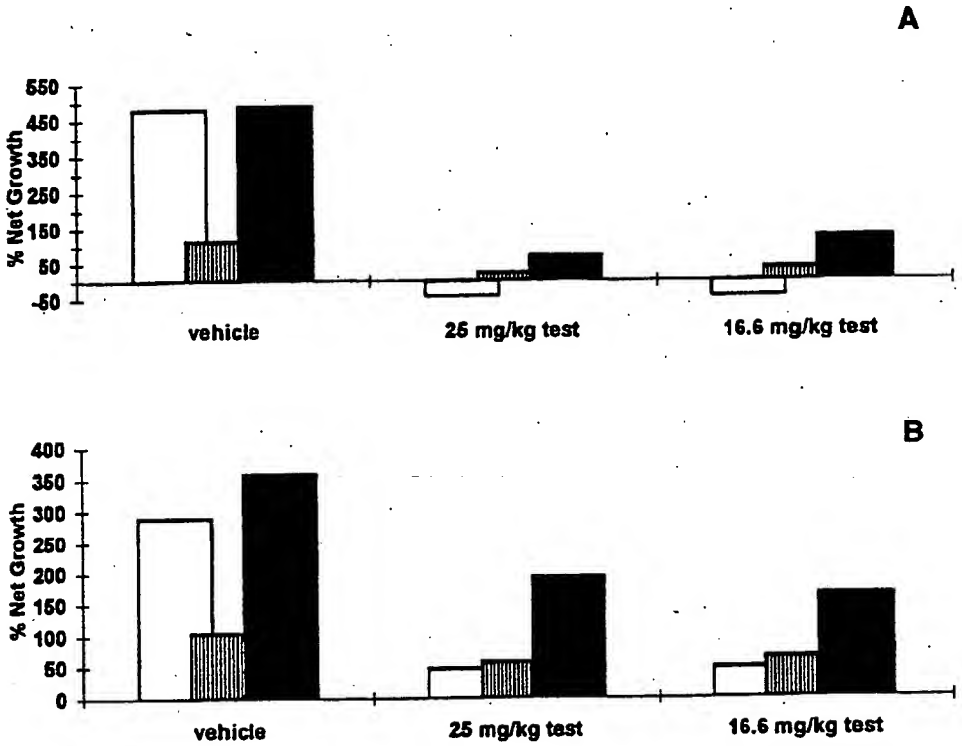

Fig. 4 In vivo effects of test agents on growth of human tumor cells in hollow fibers. Cell lines are COLO 205 colon, open bars; U251 glioblastoma, striped bars; and OVCAR-5 ovarian; solid bars. **A.** ip Implants. **B.** sc Implants. Test agents was administered ip on days 1–4, and fiber contents were assayed on day 6.

5. SUMMARY

The discovery and development of potential anticancer drugs by NCI are based on a series of sequential screening and detailed testing steps to identify new, efficacious lead compounds and to eliminate inactive and/or highly toxic materials from further consideration. Past experience in large-scale screening with a wide variety of animal and human tumor systems and the management of disease-free athymic mouse facilities has proven to be highly valuable for the recent characterization, calibration, and utilization of newly acquired human tumor xenograft models. Furthermore, DTP's experience with computer programming has enabled the development and implementation of specialized analytical software, which permits acquisition, storage, and presentation of data in readily accessible tabular and graphic formats. Many of the human tumor xenografts have been employed to test a variety of distinct chemical compound classes over the past five years. Thus, the in vivo drug sensitivity profiles of these human tumor xenografts are well suited to serve as "benchmarks" for the testing of newly synthesized agents as well as agents isolated from natural product sources that are currently under investigation.

In addition to standard models of in vivo testing, NCI has also utilized human tumor materials to explore the suitability of alternate model systems, e.g., "metastasis" and "orthotopic" models. At present, our program is investigating the utility of a capillary hollow-fiber implantation model as a means to prioritize compounds active in the in vitro screen for subsequent in vivo evaluations. It is hoped that further refinement and application of the procedures described in this chapter will facilitate the identification and preclinical development of more efficacious treatments for human malignancy.

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ANTICANCER DRUG DEVELOPMENT GUIDE

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1 Perspective on the History of Tumor Models

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"In spite of the fact that no animal tumor system has been demonstrated to reliably predict for response of any specific human tumor to drug treatment, we believe that laboratory studies indicating improved therapeutic responses in animals have been useful to clinical oncologists in improving cancer treatment in man, and the future promise of laboratory successes for indicating improved treatment of tumors of man is great" (1).[a]

1. INTRODUCTION

When I was a younger researcher working with Frank Schabel in the few years prior to his retirement and death, I would frequently join him in his office to talk science. Often he would be seated at a table with a manuscript he was reviewing spread before him. He would tap some tabulated data or a figure with his finger and then, to be certain of my attention, he would reach over with the same finger and tap me on the arm or knee and say, "We've *got* to teach 'em." Invariably, these data would be offered to support a claim of antitumor activity for a drug in a murine tumor model. Another

[a] After Frank Schabel's death in 1983, many whom he was pleased to consider his colleagues shared their perspectives on his career and contributions, but there are now fewer of us for whom Schabel's memory really lives. Although he possessed a towering ego, Schabel was a man who once humbly described himself to the author as "running along behind Howard [Skipper] with a teacup to catch the sloppings from Howard's bucket." He was in fact the genius for execution of Skipper's strategies for the cure of experimental leukemia. Dr. Vincent DeVita once introduced him as "the William Osler of mouse doctors." It is with overdue satisfaction that I dedicate this "perspective" to Frank M. Schabel, Jr., Ph.D. (1918–1983).

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researcher would be reporting tumor-growth inhibition, but when the numbers were considered critically and analyzed by the standards that Frank insisted on, it would be obvious, even to me, that the tumor burden in the group of treated mice had actually increased during the course of treatment. The nadir of tumor mass suggested a therapeutic response, but the experiment had not been adequately designed to assess it. And so it went. And so it continues.

As I consider the history of antitumor drug discovery and development and the use of tumor models to support this endeavor, I view time as divided into three eras. First, there was the cure of childhood leukemia. Next, there has been the ongoing effort to do the same for the major solid tumors. I believe that the second era is likely to be viewed in retrospect more as an era of failure than as an era of success. But it is leading us to the third era, into which I believe we are already in transition. Seeing this new era clearly from the perspective of cancer treatment is like seeing the kids' soccer game as they mature from their elementary school years to college. The younger kids focus on one thing—smashing the ball. The older kids, tutored by experience and coaching, learn to set up the shots so that when the killer blow is delivered, it scores. In much the same way, I believe that the treatment of solid tumors is moving from an approach that has focused on smashing the tumor cell with a drug or drug combination—in the best tradition of leukemia treatment—to an approach that will allow us to set up the tumor cell so that when the killer dose is delivered, regression and cure occur with satisfying frequency.

The new approach is not meant to abandon the principles that have led to the cure of some cancers. Rather, I am referring to new targets and treatment approaches that are being added to the established, well-worked strategies. But how must we approach these targets and treatments experimentally with our tumor models? Here, at the end of just over 50 yr of experimental cancer chemotherapy research, we should be able to determine with some confidence what tumor models will and will not do for us, and what they predict and don't predict. The heterogeneity of cancer continues to impose pressures on our models that are not duplicated in any other disease setting. The goal of this chapter is to call attention to some of the important lessons of the first and present eras, because it's still important to "teach 'em," while I depend on the remainder of this book to describe the advent of a new era of triumph in cancer treatment.

1.1. The Scope of Modeling Tumor Behavior

When "model" is used in the cancer literature of the past five decades, it may refer to an experimental tumor growing in a nonhuman host, often a mouse. Alternatively, and often without the distinction of a clear context, it may refer to a mathematical description of tumor growth and regression. Because the mathematics have usually been applied to observations of experimental tumors, the mathematical "model" is really a subtype of the more broadly applicable sense of "model," where the experimental tumor may yield to a mathematical description as readily as to a biologic, biochemical, or genetic description. This distinction is important to avoid confusion, and is emphasized here as needed. In regard to the modeling of tumor growth *and regression,* the insights gained by early investigators have become much clearer in the last decade—namely, the importance of the processes underlying tumor regression. Before our present-day, more widely shared vision for cytostatic therapy, tumor regression following effective and potentially curative therapy was assumed as a given. Because an increas-

Table 1
Context for Tumor Models in Biology and Therapy of Cancer

- Discovery: Screening for new drugs
- Biology: Tumor proliferation, progression, programmed cell death
- Mechanisms: Drug interaction with target
- Development: Prediction of drug efficacy and safety

ing number of new treatment approaches aim to extend the time to disease progression and to produce stable disease, tumor regression in our models and in the clinic may be less frequently the end point of choice in the future to guide drug development and to describe positive clinical outcomes. But we must remain cautious here. There is recent evidence (Von Hoff DD, personal communication) that as we have insisted on complete regressions (CRs) and cures in preclinical models, we have seen that a concomitantly higher percentage of drugs entering the clinic ultimately exhibit clinical activity. The percentage of new drugs confirmed to be active in the clinic is higher than ever before. So while I emphasize—and even insist on—the general and historical importance of tumor regression in our models, I must acknowledge the challenge of modeling and predicting cytostatic responses.

1.2. "Our Major Tool is the Tumor-Bearing Animal" (2)

The contributions of tumor models to present-day therapy have been the usual point of departure for most previous efforts to place tumor models into a historical perspective. Simply stated, the argument has been made that tumor models of the past have led us to the drugs of the present *(3,4)*. This is essentially Schabel's position in the quotation that begins this chapter *(1)*. And in the broadest sense, there is no basis to dispute this. However, under critical examination, this argument becomes less satisfying, and leads to the controversy that has surrounded the various strategies for discovering new cancer drugs (i.e., screening) since 1985. Although aspects of this controversy are important and will be examined later, the preponderance of heat over light has been distracting, and has changed the focus of the discussion. It has been easy to forget that models have provided a means to study not only the therapy of cancer, but the biology of cancer as well (Table 1). And when one has examined the predictive fidelity of tumor models, it has been easy to emphasize their apparent failure to correlate with positive clinical outcomes—i.e. cancer therapy—and to overlook the success with which they have endowed our understanding of cancer biology.

2. CURE OF CHILDHOOD LEUKEMIA

So to pick a year, what did the cancer therapy landscape look like in 1970? From the perspective of tumor models, where was the focus? Answers may be discerned in a review by Scott *(5)*, who mentioned little other than leukemias and lymphomas. Even the anthracyclines were characterized as antileukemic agents in 1970. Asparaginase was of theoretical importance. Less than three dozen active drugs were known (*see* Table 2, refs. *6,7*). Cisplatin was not known yet. And only (or predominantly) patients with leukemia or lymphoma benefited from chemotherapy.

Table 2
History of Cancer Drug Availability

Decade	*Number of currently used drugs that entered clinic*[a]	*Example*
1940	6	Methotrexate
1950	14	Fluorouracil
1960	14	Doxorubicin
1970	4	Tamoxifen
1980	16	Taxol
1990	(55)[b]	Gemcitabine

[a] Ref. 6
[b] Ref. 7

The title of this section is also the title of an important and helpful work by Laszlo *(8)*. As a historical milestone, the cure of childhood leukemia marked an era identified in the Introduction. However, as the reader will have anticipated, the subject of this section is more properly the cure of *experimental* leukemia. Laszlo captures well the entrepreneurial spirit of the day among those at the heart of the cancer chemotherapy revolution. This was a clinical revolution led by outstanding clinical investigators. But at the periphery of this revolution was a vigorous program of applied research aimed at addressing critical clinical issues with work in tumor models. And within the context reflected by Scott *(5)*, the models of the day, like the clinical targets of the day, were *leukemias*,—most notably, the L1210 developed by Lloyd Law prior to 1949 *(9)*.

When the clinical groups confronted and described any one of a series of obstacles on the path to success, the mouse doctors, who were kept in close communication with the clinicians through Zubrod's network *(8)*, would launch L1210 experiments to explore the obstacle. One example was the challenge of central nervous system (CNS) relapse among the children who achieved initial remission of their leukemia. Goldin's group at the National Cancer Institute (NCI) modeled this aspect of the natural history of acute lymphocytic leukemia *(10)*. They wanted to know which route leukemia cells take to enter the brain, and the extent to which systemic chemotherapy affected leukemia cells in the CNS. The tumor model (L1210) provided insights into biological (anatomic) as well as therapeutic (pharmacokinetic) aspects of the clinical problem.

DeVita *(11)* refers to a cardinal rule of chemotherapy that has its roots in this era. "The cardinal rule of chemotherapy—the invariable inverse relation between cell number and curability—was established in this model [L1210] and applies to all others." One important lesson of this early era is *not* that tumor models have given us this or that drug, but that tumor models, including L1210, have given us cardinal rules that have guided the advancement of clinical experimentation in cancer treatment. And without question, more of these cardinal rules are based on the work of Lasker Award winner Howard Skipper *(8)* than any other experimental chemotherapist. I have not found a finer, more succinct and critical summary and appraisal of Skipper's work than that of Jackson *(12)*. As he recounts,

> *"In 1964, Skipper and colleagues published a long paper dealing with the curability of a mouse leukemia at different stages of advancement, with a variety of single drugs. Three major conclusions were stated: (1) There is an invariable inverse rela-*

tionship between the total leukemia cell burden and curability with chemotherapy; (2) dose-response relationships are apparent for all classes of anticancer drugs and are reflected in both the survival time of treatment failures and cure rates; and (3) a given dose of a given drug will kill approximately the same percentage, not the same number, of widely different-sized leukemia cell populations—as long as their growth fraction and degree of phenotypic heterogeneity are similar."

The import of the third major conclusion warrants a subsection of its own (*see* Subhead: 2.1.).

Skipper went on to focus on the problem of drug resistance, and how the existence of or the emergence of drug-resistant clones within a tumor-cell population may account for clinical treatment failure. As stated by Jackson *(12)*, "The modeling studies of Goldie and Coldman and of Skipper provided a powerful tool for the study of why chemotherapy fails and how to prevent failure." The point is that in the era culminating in the cure of childhood leukemia, leukemia models permitted serious study of *big* questions of importance to the improvement of leukemia treatment. It is important to recognize that to this point, tumor models were more *generally* predictive for clinical outcomes vis-à-vis treatment *strategies* than they were specifically predictive for clinical activity of a particular drug in a particular setting. These accomplishments cannot be trivialized by familiarity or the passage of time. The 20 years from 1945 to 1965, when childhood leukemia was cured, saw the reduction to practice of a remarkable (at that time) concept—the drug-induced cure of a cancer. Leukemia models were important, and their contributions are hardly overshadowed by advances of the recent past two decades, impressive though they are.

2.1. Log-Kill or Log-Cell-Kill

"The Skipper-Schabel-Wilcox model or 'log-kill model' was the original, and is still the preeminent, model of tumor growth and therapeutic regression." So declared Dr. Larry Norton *(13)* in a thoughtful evaluation of clinical outcomes in breast cancer. This log-kill or log-cell-kill model was based on work in Skipper's group by Wilcox *(14)*. It is related to the third major conclusion in Skipper's work cited earlier *(12)*—that "a given dose of a given drug will kill approximately the same percentage, not the same number, of widely different-sized leukemia cell populations." Restated by their clinical counterparts (e.g., Blum and Frei, *15*), who referred to "this log cell kill concept,"

"In general, for homogeneous populations of tumor cells there is a log/linear relationship between tumor-cell destruction and dose."

Lloyd *(16)* provided a formal definition:

"The term 'log kill' is defined formally as $y = -\log(F)$ where F is the fraction of tumor surviving treatment. It provides the means of accommodating a wide dynamic range of the variable (in this instance, F) in a dimensionless quantity of convenient magnitude. For example, if the fraction surviving is 0.1, then log kill = 1.0, or if the fraction surviving is 0.0000000001, then log kill = 10.0. Not only is a log kill easily converted to the fraction surviving, but it also represents the number of log units by which the tumor is reduced. If, for instance, a tumor size of 10^{10} cells is subjected to a treatment which results in a 4.5 log kill, then the number of cells remaining is $10^{5.5}$."

The tumor model that provided the basis of the log-cell-kill concept was L1210, the same tumor model whose value was emphasized in the preceding section. But Lloyd *(16)* and others extended the applicability of log-cell-kill to solid tumors as well.

There is a misconception that the log-cell-kill concept applies *only* to leukemias. Norton may be cited *(13)* as having challenged aspects or applications of the Skipper-Schabel-Wilcox model. And indeed, when one considers the principal assumption of this model, one recognizes the limitation emphasized by Norton. The assumption is that a fixed doubling time accurately reflects the proliferative behavior of a given tumor. But Norton points out, for example, that chemotherapy may alter the cytokinetics of residual tumor cells *(13)*. The Norton-Simon hypothesis *(12,13)* states that in solid tumors growing with Gompertzian kinetics, the rate of regrowth increases as the tumor shrinks, so that cure requires greater dose intensity than induction of regression. Skipper had expected that exponential growth and a fixed doubling time would characterize all tumor-cell populations that were sufficiently small, especially micrometastases. However, Norton points out that "clinical experience, unfortunately, has not *entirely* [emphasis added] confirmed these optimistic predictions" *(13)*. The failure of adjuvant chemotherapy of breast cancer to achieve expected tumor-burden reductions required an explanation, and Skipper's was mutation of cells to a drug-resistant phenotype *(12)*. However, for our purposes, one need not conclude that the log-kill model is generally inconsistent with clinical experience—quite the contrary. The log-kill model provides a useful *estimate* of the effect of treatment on cell number in solid tumors as well as in leukemias. What the model does not account for is the failure of residual tumor cells to maintain an unchanging genetic profile. The result is that over time, the fraction of solid tumor cells killed by a given dose is *not* constant because the cells change—i.e., the cells themselves are *not* constant *(13)*.

The Skipper-Schabel-Wilcox model is so meaningful because it conceptualizes both tumor growth (exponential) and tumor regression (log-kill) in response to chemotherapy *(13)*. In my experience, one is far more likely to go wrong in interpreting experimental chemotherapy data from any tumor model by *not* applying the log-cell-kill concept than by relying on it to estimate the effect of a treatment on tumor burden in a mouse.

3. CYTOREDUCTION IN SOLID TUMORS

Just as antibacterial chemotherapy in 1945 broadly exposed the public to the concept of chemotherapeutic *cure* and raised the expectation that similar success could be ours with all diseases, including cancers, the success of L1210 leukemia as a model of acute lymphocytic leukemia in children raised the expectation, still prevalent today, that a murine solid tumor of a given histologic type will behave in a predictive way for a human solid tumor of the same histologic type. Schabel wrote:

"We clearly lack highly reliable predictive experimental tumor systems for most of the solid tumors in man..." (2),

and his words remain as accurate a characterization of our situation today as they were in 1972. It cannot be overemphasized how common is the logical but uninformed belief that response of a given histologic type of cancer—e.g. ovarian, especially of a

transplanted human tumor xenograft—in a preclinical setting is predictive or more-than-hopeful for a positive clinical outcome in the same histologic type of cancer. Why is this not the case? Schabel's assessment offers answers *(1)*. Here are four:

1. Lack of common etiology or natural history, including the program of progressive genetic changes that accompanies progressive neoplastic disease, between tumor models and human disease;
2. Ability to characterize tumors beyond histologic type is greater now than when Schabel wrote (23 years ago), but selection of the tumor cell to match the human disease does not necessarily solve the preceding etiology problem;
3. There is a tendency *not* to study advanced disease in models; and,
4. Drug exposure often differs among hosts, as does biochemistry—e.g. folate status.

So tumor models, especially solid-tumor models, have continued to exhibit important limitations. But the opposite extreme is no more fair an assessment of tumor-model usefulness than the previous one. Often, I have witnessed presentations of work in a tumor model, followed by a clinical investigator who asserts that *we all know* those models are not likely to be predictive for patient responses! Well, we most emphatically *do not* know this. We *suspect* that response in a single or a few models predicts little, with confidence, for patients. However, *as the number of responding models (by rigorous definition) increases, the confidence of predicting "some" response in patients also increases.* To quantitate or generalize this continues to be elusive, and the emphasis is on "response." It is noteworthy that in tumor models, aspects of experimental design where the end point is *not* response—such as drug kinetics or effects on a molecular target—have been far less contentious. Historically, most of the controversy that seems to have surrounded the usefulness of tumor models has focused on the issue of response.

3.1. Perspective on Predictive

Earlier, I stated that attempts to extend to common solid tumors the success that had been achieved by 1965 with leukemias and a few other high-growth-fraction tumors marked the second era in the development of cancer therapy—one that continues to the present day. This era has lacked the signal success(es) of its predecessor—a point not likely to be disputed. But what have we learned about tumor models in the past 35 years? Schabel made a compelling case, often ignored, for using tumor models in a way that reflects as closely as possible the realities of the clinical situation *(1)*. Dedrick has pointed out that "...the mouse is not just a little person" *(17)*. And the model is more than just the mouse. Realities of the clinical situation include orthotopic disease, advanced disease, disseminated disease, host metabolism, host kinetics, and a host of other variables in addition to the heterogeneity of the tumor-cell population, with all its implications.

With this in mind, what do the authorities say about the predictive reliability of tumor models? I have already cited Schabel *(1,2)*. Martin et al. wrote, "Many clinical investigators believe that murine tumor models are not relevant to the human cancer problem..." *(18)*. Grindey stated: "While none of the in vivo models currently available directly predict for overall clinical utility, they do provide very important information required for assessing probable clinical potential" *(19)*. And Fidler *(20)* said "No" to both of the following questions:

1. Are experimental tumor models predictive for therapeutic response of human cancer?
2. Is one tumor model predictive for response of a second tumor model?

Venditti went even farther: "...patients bearing tumors of a given type do not necessarily provide a perfectly predictive model for others with the same tumor type" *(21)*. But as Martin et al. pointed out, "The predictive value of animal tumor models could be readily improved simply by imposing more rigorous criteria for the selection of clinical candidates" *(18)*. And I might add that this is true regardless of whether the context is screening or more advanced drug development. But what *criteria? Response* criteria. We must consider, as an important aspect of the model, how we define "response." What demands should we make of our tumor models? Schabel *(1)*, Martin *(18)* and others would have us look at *clinical* criteria for response. And we shall. But unfortunately, we must recognize that even here there is a lack of uniformity.

Tonkin, Tritchler, and Tannock *(22)* analyzed the standard of practice for defining tumor response in clinical trials in 1985. They found that the criteria for tumor response were poorly defined and variable, and that differences in response criteria contributed to variability in reported response rates. One of the reasons that one patient seems to be a poor model for the next *(21)* is suggested here—lack of uniform end points for tumor response to treatment. Tonkin et al. noted that there was disparity in reported responses of patients with the same cancer treated with the same regimens. They found "heterogeneity in the criteria that are used by different investigators to determine tumor response. ... it is evident that 'standard' criteria [of response] do not exist" *(22)*. Although "...all investigators require >50% shrinkage in cross-sectional area for partial response," even this is not without some variability *(22)*. For our purposes, there are two important points here. First, the clinicians themselves do not adhere uniformly to standard definitions of end points, complicating but not excusing our own shortcomings in applying "rigorous criteria" to the design and interpretation of experimental trials aimed at predicting clinical activity. Second, by 1985, certain definitions were at least *accepted* on a fairly broad scale, including "partial response" (PR) and "complete response" (CR). The case has been made eloquently and emphatically that these end points are the measures of choice for tumor models as well as for human cancer *(1,18)*. Recall that the log-cell-kill concept was worked out for leukemia, but that Lloyd *(16)* and others extended its applicability to solid tumors. Later I will examine how log cell-kill might best be used in this context. However, anyone uncomfortable with log cell-kill can have no complaint about recording >50% regression of measurable tumors. It is noteworthy that if one measures individual tumors in mice twice per week for an adequate time, both end points are available from the same data. One end point (PR or CR) provides clinically relevant and understandable information. The other (log cell-kill) permits estimation of a quantitative effect of treatment on a tumor-cell population that responded, perhaps with delayed growth, but did not regress sufficiently to qualify as a PR or CR, a population for which one may wish nevertheless to compare effects of two or more treatment regimens.

3.2. The Take-Home Message

If we wish to improve the predictive reliability of our solid-tumor models for drug activity in human cancer, we must certainly demand more rigorous criteria for declar-

ing a drug to be active in the preclinical setting *(1,18)*. And in order to make decisions about the development of a new drug, what criteria must be applied? "Mere inhibition of solid tumor growth is usually used as the criterion for activity in the preclinical setting, whereas 50% or greater tumor regression, *requiring the killing of 2 or more logs of the clonogenic tumor cells* (emphasis added), is the acceptable criterion for activity in clinical trials" *(18)*. *That's pretty clear.*

> *Laboratory workers have reported anticancer treatment of animal tumors as being positive, and therefore effective by inference, if drug treatment held the growth of solid tumors to 50% or less of that of untreated controls. Even complete inhibition of tumor growth under treatment would be negative to the clinician, since he requires reduction of the tumor mass by greater than 50% for objective [cytoreductive] activity by drug treatment (23).*

If I am developing an antitumor drug and I am at any stage along the value chain from discovery to investigational new drug (IND) application and I want to say something about the activity of my drug, here's what I need to be saying. Partial regression (PR) of a tumor model is good. Complete regression (CR) is better. Cure—the disappearance of an established tumor and its failure to regrow during an observation period equal to several volume doubling times after cessation of treatment—is best. And the more models in which I do this, the better. Finally, a two-log reduction in tumor burden at the end of treatment is the minimally acceptable definition of antitumor activity when the expectation is cytoreduction. When the expectation is that the drug will produce cytostasis, one must show, at a minimum, that the tumor burden did not *increase* during the treatment period. Both conclusions require the same tumor staging at the start of treatment and the same serial tumor measurements during the course of the experiment.

The origin of this "two-log" rule is a bit difficult to determine. Martin et al. *(18)* mentioned it. Corbett *(24)* has employed it. It probably goes back again to the leukemia work. Blum and Frei *(15)* defined PR and then commented on the quantitative cell-kill (in logs) required. Although their context was drug combinations and the drug effects (cell-kill) required for therapeutic synergism, their correlation of log-kill and response is clear. A one-log reduction in the tumor burden in a host, man or mouse, will appear as a PR in leukemia. Whether it will be measurable as a PR in a solid tumor will depend on the kinetics of regression in that tumor and its host *(25)*. To effect a CR (in leukemia), at least a two-log reduction in tumor-cell number is required. If one observes a CR in a tumor model, at least two logs of cells will have been killed. If the kinetics of regression of a solid-tumor model are such that one only measures a delay in tumor growth, this delay (in days) may still be used to estimate log cell-kill. And if the log-kill is two or more, one may conclude that a CR is probably attainable, perhaps with extended treatment. James et al. *(26)* have estimated that a 50% reduction of tumor *diameter* equates to a tumor-volume reduction of 87% or a *tumor burden reduction of about one log.* Parenthetically, the paper by James et al. *(26)* serves to update the conclusions of Tonkin et al. *(22)* mentioned earlier, and there is still no standard for clinical response criteria, although World Health Organization (WHO) criteria are described as "most frequently used" now. However, experimental oncologists, faced with the challenges of new drug mechanisms, cannot afford to ignore standards for defining drug activity. The effect of treatment on tumor burden must be estimated and interpreted in tumor models at the end of treatment,

and the minimum acceptable response suggesting clinically relevant drug activity is a two-log reduction of tumor burden, with CRs and prolonged tumor-free survival (cure) as the goal. "The accurate conversion of appropriate tumor measurement endpoints or animal survival data into $\log_{10}$ tumor cell kill is essential for any meaningful evaluation of therapeutic effectiveness. In chemotherapy experiments with syngeneic tumor models, changes induced in tumor volume doubling time are rare. Thus, with correctly staged and well-controlled chemotherapy experiments, there is little trouble in the conversion of appropriate observable endpoints into $\log_{10}$ tumor cell kill" *(27)*. Elsewhere, Corbett *(24)* concluded that "...the more tumors [tumor models] that respond, respond at more than one dose level, and respond markedly [>2-log kill, CR, or cure], the more likely the agent will be clinically useful." And Johnson *(28)* observed that "...drugs with a high degree of efficacy and a broad spectrum of activity in animal tumor models have generally proven to be useful in the treatment of human cancer." Indeed, said Grindey, "...it seems reasonable that therapeutic approaches [drugs] with a broad spectrum of antitumor activity in the preclinical tumor model might have the best clinical potential" *(19)*.

3.3. The Search for New Drugs Active Against Solid Tumors

In the so-called screening controversy surrounding the discovery of new cancer drugs, my primary purpose is to re-emphasize that the perspective I wanted to capture here has less to do with using tumor models to detect or discover or screen for new drug activity, and more to do with using tumor models to make decisions about advancing to the clinic with a drug believed to have antitumor activity. Nevertheless, it was March 1980 when a watershed conference was held at Airlie House in Virginia to assess the state of tumor models *(19–21)*. Partly because of the influence of that conference, by 1985 important changes were in place in the NCI drug screening program *(29)*. These changes were sufficiently profound to precipitate a number of the articles from which this present perspective has been drawn *(4,18,28)*, and others that provide additional insight *(30)*, not to mention seemingly endless discussion. Most readers know this story. The NCI screen had been an in vivo screen composed of murine leukemia, syngeneic murine solid tumors, and three human tumor xenografts. It was replaced by an in vitro screen of 60 tumor-cell lines representing major human cancers for which new therapies were (and are still) needed *(29)*. A secondary screen was eventually elaborated, consisting of solid-tumor xenografts derived from the 60 cultured-cell lines *(31)*. And the clock began to run. That was 15 years ago. No one has published a definitive or conclusive evaluation of the 15-yr screening experience with the 60 cell lines. I can think of no clinically active drug whose discovery was linked exclusively to the NCI program during this period, but it may be too early. This is only one opinion and one recollection—mine. But this is *not* the point of the present perspective. New drugs can be discovered in numerous ways. To succeed in human patients, a new drug must modify the course of the disease it is intended to treat. Modifying the course of cancer can be tested in available tumor models. And if appropriate end points are used in the preclinical setting, experience suggests that the likelihood of seeing clinical activity can be maximized. However, it must be borne in mind that no amount of confidence in our models

> *...will save the experimental and clinical investigator the pain of having to learn to 'translate' (emphasis added) in both directions" (18). "In the present context, transla-*

tion means the changes in regimen required to achieve a similar response in a second tumor system when a given response to some therapeutic procedure is observed in one tumor system... (32).

Translational research will be discussed further in a later section.

3.4. An Example—Quinocarmycin

It is noteworthy that most published descriptions of tumor models only *describe* the models or their evolution. They generally *do not* document "track record" or answer the questions: For what are they useful? What will they tell us? What do they predict? How well do they predict it? If it turns out that drug candidates taken into clinical development lack activity, why might this be? Did our tumor models predict inaccurately? The search for new cancer drugs—particularly for disease-specific drugs—over the past 15 years has yielded opportunities to explore this question. Drawing from the published NCI experience, the clearest example may be the quinocarmycin analog DX-52-1 *(33)*.

This agent represents one of the first to be selected for preclinical development based on disease-panel specificity [melanoma] discovered in the National Cancer Institute cancer drug screen (33).

Examination of a more detailed presentation of the tumor model data two years later *(34)* is instructive. In this later article, Plowman et al. indicate that DX-52-1 "...demonstrated statistically significant antitumor activity against five of seven melanoma xenografts" *(34)*, but this activity was measured as *tumor-growth inhibition.* Only two of seven melanoma xenografts responded with clinically relevant PRs or CRs. Of seven xenograft models derived from cell lines that were sensitive to DX-52-1 in vitro, one would want at least four models to respond with PRs or CRs in order for the drug to be accepted as active. So what has the clinical experience revealed? Herein lies an example of a great historic weakness in the way cancer drugs have been developed—coordination of clinical trial design with data from preclinical models. Public databases indicate (and personal communications confirm) that DX-52-1 was studied in the United States in two NCI-sponsored Phase I trials. Although the results are not yet public, available information indicates that *only one melanoma patient was enrolled.* It is presumed that specificity for melanoma will be tested in Phase II. Venditti et al. indicated in 1984 that our progress in understanding the prediction of activity in a specific histologic type of cancer had been hampered by limitations in the *clinical* data for new agents. Overcoming these limitations "...will require considerable clinical feedback on new agents entering clinical trial since 1976" *(3)* and to the present day. Activity in a tumor type in vitro and in vivo has not been shown to correlate with tumor-type activity (much less, specificity) in humans. In the main, this is more a failure to obtain the clinical data than an overall failure of an adequate test of the hypothesis. Where the clinical comparison *has* been done, the correlation looks surprisingly good *(35)*.

So even "...the first [agent] to be selected for preclinical development based on disease-panel specificity" (DX-52-1) and forwarded to the clinic on the basis of xenograft "responses," seems not to have received an early evaluation in a melanoma setting where disease specificity might have achieved preliminary support along with safety and pharmacokinetics. And no early indication of clinical activity has been published.

Perhaps DX-52-1 will eventually prove to be active. But in the same period (1995–2000), other agents such as ecteinascidin and cryptophycin have entered the clinic supported by much stronger tumor-model data *(36,37)* than that presented for DX-52-1 *(33,34)*. For example, in a study of cryptophycin-8, Corbett et al. reported that four of six xenograft models yielded PRs or CRs *(37)*. The other of these drugs, ET-743 (ecteinascidin), which produced PRs or CRs in two of three xenograft models in one report *(36)*, has already demonstrated meaningful clinical activity *(38)*. These examples are offered to illustrate my point, not to prove it. Greater persuasion would be beyond the scope of this perspective, and the data simply are not available. But there has been little written in the last decade to remind us of earlier thinking about how we might best use our tumor models. Disappointing as it may be, progress has not been as robust as the models might have allowed. We *must* demand the same responses from our tumor models that we want to see in the clinic. However, these are labor-intensive, expensive preclinical trials to conduct, and experience is clear that many investigators prefer to measure tumor growth inhibition as their primary end point in assessing drug activity in tumor models. Which brings me back to Von Hoff's observation (personal communication): to the extent that we have insisted on CRs and cures in tumor models, we are seeing correspondingly more positive clinical results with new drugs!

4. TARGET-DIRECTED CANCER THERAPY

> *"A new era of cancer research dawned in 1984 when mice reared from eggs injected with genes implicated in cancer (oncogenes) proved to develop specific types of tumors" (39).*

This is the third era identified earlier, into which I believe we are now moving. It is marked by new and distinctive tumor models, and by new challenges for their prudent use. By 1985, cancer-drug discovery programs were increasing in number among the major pharmaceutical companies, and more emphasis was being placed on mechanistically driven screens. The NCI responded with the National Cooperative Drug Discovery Grant program, and most of these grants focused on a discrete target, such as topoisomerase I or II, or on a molecular mechanism, such as calcium signaling. The advent of genomics has brought even higher resolution to the characterization of molecular targets. Research has focused increasingly on target validation, as practiced in the pharmaceutical industry, or target "credentialing" as referred to by Wittes and Klausner *(40)*. Target validation requires the stepwise linkage of target with disease by three critical tests. First, is the target associated with the disease, and what is the frequency of that association? Second, is the target linked mechanistically to the disease so that—to cite an increasingly common approach—knocking in, knocking out, or knocking down the target has the expected result regarding presence or absence of the disease? And third, does pharmacologic modulation of the target, at an appropriate level of specificity, modify the disease? Target validation begins at the cellular level, moves through tumor models in animals, and culminates in translation to the clinic.

Translational research *(41)* is the clinical extension of target validation, as just described. The current emphasis on translational research has grown out of the imperative *(42)* to show, in patients, the target effects of target-based drugs. From the preclinical end, the problem is more straightforward. Relevant tissues for target assessment are more readily available than in the clinical situation. Pharmacodynamic/pharmaco-

kinetic modeling in animals offers a rational reference to guide clinical trial design. From the clinical end, tissues for target assessment are more problematic, and surrogate tissues and even surrogate markers or assays are often the only resort. I suspect that these problems will ultimately yield to the forward progress of genomics. But the take-home message with regard to the tumor model is not the choice of target, not the assessment of the target, and not the extrapolation of pharmacokinetics across species. The take-home message from the preclinical end of translational research is that *translation will be flawed unless the pharmacodynamic piece* (therapeutic response) *is based on clinically relevant response criteria.*

After more than a quarter of a century of engagement with the cancer problem, the one lesson that seems to me to stand above all others is that each new layer of complexity foreshadows the next layer of greater complexity. We are now embracing the notion that if we can demonstrate target modulation in a tumor model, this will more reliably indicate a positive clinical outcome. But the "next layer" seems to challenge the notion that therapy directed to a single target will have a meaningful impact on cancer in patients. "Tumor cells represent a highly evolving population of cells with multiple potential targets. Any cancer treatment must start with this recognition. It is unlikely that drugs aimed solely at a single target, however important it may be, would be effective in cancer management" *(43)*. Zhang et al. *(44)* looked at differential expression of genes, a possible clue to altered function and the targets involved, and found about 500 differences when they compared normal colon epithelium and primary human colon cancers. Stoler et al. *(45)* analyzed the extent of genetic mutations in colon cancer, which suggests the scope of genomic events that underlie malignancy, and found approx 10^4 such events per colon-cancer cell—an unexpected (at least by me) magnitude of genomic heterogeneity. How can we take the next step to target-directed therapy while assuring that our tumor models, in the face of such complexity, do not lead us into a new era of clinical disappointment?

Retain has pointed out that "for target-based anticancer drugs, it is critical to establish that the observed preclinical activity can be attributed to modulation of the target" *(42)*. Although a measurable effect on a molecular target has become a more critical piece of any proof-of-principle, an effect on a target in a tumor model may predict no better for clinical activity than our historical experience *unless* target effects are accompanied by robust therapeutic responses (CRs and cures) in the model. If single-agent activity fails to meet this requirement, then single-agent cytostasis, where one can show, at a minimum, that the tumor burden did not *increase* during the treatment period, will be imperative. Evidence of CRs and cures must then be obtained from studies of drug combinations. The question has been raised whether growth delay in xenografts will translate to stable disease in the clinic. It may be stated emphatically that if the answer is to be "Yes," the number of days of growth delay *must* equate to a net zero increase in the tumor burden over the treatment or observation period. We now need to develop data that will test the hypothesis that the longer the period during which the tumor burden remains static in xenografts, the higher the probability of observing stable disease in the clinic.

Can I illustrate what I mean by a tumor burden that, at a minimum, does not *increase* during treatment, i.e. remains static? It seems simple enough, but here are three examples of what one might encounter. Consider first a report of the preclinical activity of the MAP/ERK Kinase-1 (MEK) inhibitor, PD184352 *(46)*. The desired effect on the

target is well-supported. Then one comes to the question of therapeutic response in a tumor model (their Fig. 5). In one of three experiments, they implanted murine colon-tumor fragments subcutaneously in syngeneic mice on d 0. Mice were treated orally every 12 h on d 1–14. Tumors were measured from the day they became palpable until d 17, when the experiment was terminated. The diluent-treated controls evidenced a tumor volume doubling time of 4 d and reached an equivalent mass of 1000 mg by d 14. It is obvious from inspection that treatment inhibited tumor growth, but that tumors in treated mice had begun to grow during treatment. In this example, quantitation of tumor growth delay *(1)* and estimation of the effect of treatment on tumor burden are not possible, because the observation period was not of sufficient length. Treatment would be required to have delayed tumors from reaching 1000 mg until d 27 to reflect a net change in tumor burden of zero (cytostatis) over the 14-d course of treatment. For the sake of further illustration, a one-log *reduction* of tumor burden in this experiment (which is a little more theoretical because the implanted fragments were small on the day of first treatment) would have resulted in an additional delay until d 40 for treated tumors to reach 1000 mg. It is obvious from inspection of the published data *(46)* and from these considerations that tumor measurements beyond d 17 would have been instructive.

Consider now the activity of CGP41251, a staurosporine analog that inhibits several protein kinase C subtypes. This molecule has shown "...for the first time that a PKC inhibitor can block in vivo cell signaling pathways in cancer patients" *(47)*. If one takes a look at some tumor-model data *(48)* for CGP41251, one finds the claim of "stable disease." It is apparent from inspection of these data (their Fig. 2) that tumor volume neither increased *during* the 10-d treatment period with the higher dosage *nor* during the subsequent 9-d observation period in a bladder carcinoma xenograft model. The vehicle-treated controls evidenced a tumor volume doubling time of 7 d. Again, calculation of the effect of treatment on tumor burden is not possible, because the observation period was too short. The experiment was terminated before tumors treated with the higher dosage evidenced any growth at all, and tumor growth *delay* is the parameter that must be measured, the parameter linked to tumor-burden change *(1)*. This is seen perhaps more clearly in the final example. In this example, an inhibitor of the protein kinase C beta isoform was evaluated in an hepatocellular carcinoma xenograft model engineered to include the tetracycline-inducible promoter upstream from the vascular endothelial growth factor (VEGF) gene *(49)*. This model permitted the demonstration "...for the first time that PKC, especially the β-isoform, is a major regulator of VEGF-mediated tumor development and angiogenesis" *(49)*. If we look at the therapeutic response data, their Fig. 2 reports a statistically significant difference in growth between treated and control xenografts. Mice were treated daily beginning either on the day of tumor implantation (d 0) or on d 18 and continuing for the 46-d duration of the experiment. Did the tumor burden increase during treatment? From inspection of the data, it is obvious that the tumor volume increased throughout the course of the experiment regardless of treatment. Calculation of the log change in tumor burden reveals that the increase was about one log at d 39.

So here were three reports in which the target was convincingly affected, but where the therapeutic response data were either insufficient to interpret *(46,48)* or fell short *(49)* of the *minimum* criterion suggested here for a claim of cytostasis in a tumor model. And these models were all transplanted tumors, not "real cancer" as

judged by Klausner *(40)*. A xenograft in an immunodeficient mouse cannot be rationalized as reflecting, with more than rudimentary fidelity, a human cancer growing in its human host. But transgenic models are not ideal either. For example, "...the phenotypes of genetically engineered mice cannot be easily extrapolated to humans" *(50)*. And yet, "although genetic mutations in mouse and humans do not always lead to the same tumour spectrum, the underlying molecular mechanisms are frequently relevant to both species" *(51)*. As set forth in this perspective, experience indicates that xenograft models and newer, transgenic models *can* provide important and even predictive information for those who are making decisions about the development of cancer drugs. As Fidler has insisted, "the failure of animal tumor systems to serve as predictive models for human cancer *does not diminish* (emphasis added) their usefulness but indicates the absolute necessity to precisely define the question that the model will be used to answer" *(20)*. And as I have tried to remind us, looking forward from the foundations laid by Skipper, Schabel, Goldin, Martin, Frei, Freireich, and others, there is above all the absolute necessity to demand therapeutic responses in our tumor models that are as similar as possible to the responses that we expect to obtain clinically with new cancer treatments in the era of hope and challenge that lies ahead.

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A comparison of the in vivo and in vitro radiation response of three human cervix carcinomas.

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The radiation response of three carcinoma of the cervix tumours has been compared in vivo in xenografts using growth delay and in vitro by means of a monolayer-based clonogenic assay. Tumours have been irradiated with 60Co gamma-rays at both high (70-100 cGy/min) and continuous low dose rates (3-5 cGy/min) in order tc determine the relative in vivo and in vitro sparing effects associated with lowering radiation dose rate. In vitro, two of the lines (HX155c and HX156c) showed significant low dose rate sparing when compared to the HX160c line (which showed little sparing) ($p = 0.012$). Despite greater scatter for the in vivo data, there was a general tendency for the in vivo results to follow that predicted from the in vitro experiments. In vivo, HX156 exhibited significant sparing ($p = 0.011$) whereas HX160 again exhibited no significant sparing ($p = 0.15$). Although the HX155 line did show some sparing in vivo this did not reach significance ($p = 0.111$). All three lines showed less actual specific growth delay in vivo than that predicted from in vitro data. These in vitro/in vivo findings lead to the conclusion that rapid predictive testing of radiosensitivity (ideally utilising low dose rate irradiation) would be beneficial in determining the choice of radiotherapy regimens for the treatment of this disease.

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1: Cancer Chemother Pharmacol. 1992;30(1):43-50.

Human ovarian-carcinoma cell lines and companion xenografts: a disease-oriented approach to new platinum anticancer drug discovery.

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A disease-oriented approach to the discovery of novel platinum anticancer drugs has been established through the setting up of parallel human ovarian-carcinoma cell lines and xenografts. The correlation between in vitro and in vivo antitumour activity was determined for four reference platinum agents (cisplatin, carboplatin, iproplatin and tetraplatin) in eight companion lines. Two methods of assessing antitumour effect were used in vitro (tritiated thymidine incorporation and sulforhodamine B staining) and three were applied in vivo [28-day treated/control (T/C) ratio, growth delay and specific growth delay]. In vitro, large differences in cytotoxicity across the cell lines were observed for each drug. This was also reflected in the xenografts for cisplatin and carboplatin and, to a lesser extent, for iproplatin. A correlation analysis of in vitro vs in vivo data revealed a high, statistically significant positive correlation for cisplatin and a strong positive correlation for carboplatin. However, for the two platinum(IV) drugs, the correlation was less good. In particular, tetraplatin was markedly less active in vivo (showing a general lack of activity against all of the tumour lines) than its in vitro potency against the cell lines predicted, resulting in poor correlation coefficients. These human tumour panels may be valuable for the elucidation of both cellular/molecular and corresponding in vivo pharmacological mechanisms of platinum drug resistance Moreover, the HX/62 and SKOV-3 tumour lines, which exhibit a level of intrinsic resistance to the four reference agents both in vitro and in vivo (and which were derived from patients who had not received prior platinum therapy), represent particularly useful evaluation models for the discovery of novel broad-spectrum platinum drugs.

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Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials

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Summary. An analysis of the activity of compounds tested in pre-clinical in vivo and in vitro assays by the National Cancer Institute's Developmental Therapeutics Program was performed. For 39 agents with both xenograft data and Phase II clinical trials results available, in vivo activity in a particular histology in a tumour model did not closely correlate with activity in the same human cancer histology, casting doubt on the correspondence of the pre-clinical models to clinical results. However, for compounds with in vivo activity in at least one-third of tested xenograft models, there was correlation with ultimate activity in at least some Phase II trials. Thus, an efficient means of predicting activity in vivo models remains desirable for compounds with anti-proliferative activity in vitro. For 564 compounds tested in the hollow fibre assay which were also tested against in vivo tumour models, the likelihood of finding xenograft activity in at least one-third of the in vivo models tested rose with increasing intraperitoneal hollow fibre activity, from 8% for all compounds tested to 20% in agents with evidence of response in more than 6 intraperitoneal fibres ($P < 0.0001$). Intraperitoneal hollow fibre activity was also found to be a better predictor of xenograft activity than either subcutaneous hollow fibre activity or intraperitoneal plus subcutaneous activity combined. Since hollow fibre activity was a useful indicator of potential in vivo response, correlates with hollow fibre activity were examined for 2304 compounds tested in both the NCI 60 cell line in vitro cancer drug screen and hollow fibre assay. A positive correlation was found for histologic selectivity between in vitro and hollow fibre responses. The most striking correlation was between potency in the 60 cell line screen and hollow fibre activity; 56% of compounds with mean 50% growth inhibition below $10^{-7.5}$ M were active in more than 6 intraperitoneal fibres whereas only 4% of compounds with a potency of 10^{-4} M achieved the same level of hollow fibre activity ($P < 0.0001$). Structural parameters of the drugs analysed included compound molecular weight and hydrogen-bonding factors, both of which were found to be predictive of hollow fibre activity. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: anticancer drug discovery; in vitro-to-in vivo correlations; clinical trials

Since its inception in 1955, the National Cancer Institute's (NCI) Developmental Therapeutics Program (DTP) has utilized various experimental screening models to select agents for evaluation as clinical candidates. The philosophical position from which this endeavour proceeded was that elucidation of empirically defined anti-tumor activity in a model would translate into some likelihood of activity in human cancer. The choice of specific screening models was based primarily on response of the models to agents already identified as clinically active (Gellhorn and Hirschberg, 1955; Zubrod et al, 1966). Initially, 3 transplanted rodent models were used: Sarcoma 180, Carcinoma 755 and Leukaemia L1210. The spectrum of models was then broadened, retaining L1210, which was discerned to be the most predictive of clinical activity, and adding a series of transplanted rodent models. This scheme was replaced in 1975 by the murine P388 leukaemia model, which was utilized as a pre-screen and followed by a panel of tumours. This panel first included only rodent tumours but was later enhanced to include human tumour xenografts (Venditti, 1981; Venditti et al, 1984). The human tumour xenografts were employed with the intent of their serving as potentially better predictors of clinical activity against solid human tumours.

In early 1990, the P388 pre-screen was replaced by an in vitro human tumour cell line assay comprised of 60 different cell types (Alley et al, 1988; Monks et al, 1991; Paull et al, 1995). Agents selected on the basis of potency, selective activity against a particular disease category, and/or differential activity against a few specific cell lines were then evaluated against a small number of sensitive human tumours in the nude mouse xenograft model (Dykes et al, 1992; Plowman et al, 1997) as a basis for selecting compounds for further preclinical development. Owing to the large numbers of molecules emerging from the in vitro screen as candidates for xenograft testing, in 1995 this development path was further modified to include a hollow fibre (HF) assay (Hollingshead et al, 1999), activity in which was a prerequisite for study in classical xenograft models. The HF model, where cells are introduced from tissue culture into semi-permeable fibres in mouse intraperitoneal (i.p.) or subcutaneous (s.c.) space and exposed to test agents, is a rapid and efficient means of selecting compounds with the potential for in vivo activity in conventional xenografts in pilot and 'training' sets of compounds.

This drug screening and development scheme remains an empirical one, as compounds are prioritized for development based on the definition of anti-proliferative in vitro and in vivo responses. Owing to an emerging understanding of the molecular

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basis for human cancer, great interest exists in transitioning from an empirical to a potentially more rational, molecular-targeted approach to the discovery and development of novel cancer therapeutics (Sausville and Feigal, 1999). Of particular interest will be the design of models to detect the action of compounds on particular predefined targets. The 'performance features' of compounds that have been evaluated in the 'empirical' development scheme may be of value in serving as a baseline against which newer compounds and models may be compared.

To this end, we present here an experience attempting to correlate activity in the clinic with antecedent activity in preclinical models. In addition, we present NCI's cumulative experience with the performance of the HF assay in relation to in vitro activity and to certain chemical characteristics of the compounds.

MATERIALS AND METHODS

Agents used

Data for 39 agents which had completed phase II trials (Table 1) and which had also been evaluated against in vivo tumour models were compiled. 13 of these compounds were 'standard' agents and the remaining 26 were compounds for which NCI INDs were filed between 1980 and 1996.

Table 1 NSC numbers and common names for 39 phase II clinical agents

NSC Number	Common name
740	Methotrexate
3053	Actinomycin-D
3088	Chlorambucil
8806	Melphalan
19893	5-FU
26271	Cyclophosphamide
26980	Mitomycin C
45388	Dacarbazine
49842	Vinblastine
105014	2-CDA
119875	Cisplatin
123127	Adriamycin HCL
125066	Bleomycin
125973	Paclitaxel
141633	Homoharringtonine
172112	Spiromustine
253272	Carecemide
264880	Dihydro-5-azacytidine
267469	Deoxydoxorubicin
269148	Menogaril
281272	Fazarabine
286193	Tiazofurin
308847	Amonafide
312887	Fludarabine phosphate
325319	Didemnin B
332598	Rhizoxin
336628	Merbarone
337766	Bisantrene
339004	Chloroquinoxaline sulfonamide
347512	Flavone acetic acid
349174	Piroxantrone hydrochloride
352122	Trimetrexate
356894	Deoxyspergualin
361456	Pyrazine diazohydroxide
366140	Pyrazoloacridine
409962	BCNU
609699	Hycamptamine
616348	CPT-11 (irinotecan)
628503	Taxotere (docetaxel)

For an analysis of factors affecting xenograft outcome, 1228 compounds for which both current xenograft data and activity in NCI's 60 cell line in vitro anti-cancer drug screen were available. The compounds were tested in a median of 5 xenograft experiments (range 1–131) and a median of 4 different histologies (range 1–14). In addition, 564 of these had been evaluated in the HF assay. Compounds were selected for xenograft testing at least partially on the basis of performance in the 60 cell line assay and/or the HF assay. Of these, 756 are open, publicly available compounds and 472 are discreet, confidential structures.

A set of 2304 compounds which was evaluated in the 60 cell line assay (1252 open, 1052 discreet) and referred for evaluation and testing in the HF assay was analysed. The compounds represented a wide variety of structural types, and ranged in in vitro potency from GI_{50}s (concentration of drug which achieved 50% growth inhibition averaged over all 60 cell lines) of 100 μM to less than 10 nM. Reasons for referring the compounds for HF evaluation included selective activity against cell lines of a particular histologic type, 'non-standard' mechanism of action as determined by the COMPARE pattern recognition algorithm (Paull et al, 1989), as well as compounds which were structurally novel and possibly representing novel chemotypes directed against defined mechanisms (e.g. topoisomerase inhibition). Lists of the non-proprietary compounds in these data sets are available at http://dtp.nci.nih.gov/docs/bjcwebsup.html.

Clinical response

Clinical response rate of particular histologic tumour types to the 26 NCI IND agents was reviewed in patients from all completed phase II trials with at least 9 patients; the trials were conducted under NCI sponsorship or otherwise included in Cancer Therapy Evaluation Program records, and were carried out with appropriate ethical committee approval. A 'positive' phase II trial involved an objective 50% reduction in tumour size in at least 20% of patients. For the 13 standard agents, response rates were taken from DeVita (De Vita et al, 1982, 1985, 1989, 1993, 1997). All abstractions were done by professional abstractors from EMMES Corporation (Potomac, MD).

Preclinical models

Compounds were tested in a variety of xenograft models according to methods described by Dykes and Plowman (Dykes et al, 1992; Plowman et al, 1997). Two levels of response were considered. For 'survival' models, the 2 thresholds were increased life span of 25% or 50%. In other subcutaneous xenograft models, estimation of tumour weight allowed calculation of treated/control weight ratios (T/C), with thresholds of 40% and 10% indicative of a level or degree of activity in this analysis. Agents were in all cases studied at or below the maximum tolerated dose, defined as the greatest quantity of test agent given as an acute dose which a mouse is able to survive for 11 days.

Agents were tested in the HF assay as described by Hollingshead (Hollingshead et al, 1999). A standard panel of 12 tumour cell lines are used for routine HF screening, including non-small cell lung carcinoma lines NCI-H23 and NCI-H522, breast carcinoma lines MDA-MB-231 and MDA-MB-435, colon sarcoma lines SW-620 and COLO 205, melanoma lines LOX and

UACC-62, ovarian carcinoma lines OVCAR-3 and OVCAR-5, and glioma lines U251 and SF-295. The cells, at densities of 2–10 × 10^6 cells ml^{-1} are flushed into polyvinylidine fluoride fibres having an internal diameter of 1 mm. The fibres are heat-sealed at 2 cm intervals, and the samples are placed into tissue culture medium and incubated for 24 to 48 hours prior to implantation. On the day of implantation, samples of each tumour cell line preparation are quantitated for viable cell mass by a stable endpoint MTT assay so that the time zero cell mass is known. Each mouse receives 3 i.p. implants representing 3 of the tumour cell lines, and 3 s.c. implants of the same 3 tumour cell lines. Mice are treated with experimental agents, which have been solubilized using 10% DMSO in saline and tween 80 (0.05%), on day 3 or 4 following fibre implantation and continuing daily for 4 days. Each agent is administered by i.p. injection at 2 dose levels. The dose levels are determined from the single dose i.p. maximum tolerated dose (MTD) for each test agent; the high dose being the MTD × 0.375 and the low dose being the MTD × 0.25. The fibres are collected from the mice on the day following the fourth treatment and viable cells estimated by the MTT assay. A 50% or greater reduction in percent net growth in the treated samples compared to the vehicle control samples is considered a positive result. A total of 48 fibres is treated in a standard experiment (12 cell lines × 2 implant sites × 2 dose levels). Each treated fibre exhibiting at least a 50% net reduction in cell growth is assigned an arbitrary score of 2 points, and the number of fibres reaching this level in the i.p. and s.c. compartments are recorded separately. Any cell lines in which cell kill is observed are also recorded.

Criteria were initially established for compound activity using a training set of 80 randomly selected compounds which were evaluated in both the HF and xenograft assays. The goal of the training set was to define a scoring system which allowed bias in favour of detecting all compounds with xenograft activity, defined as ≤40% T/C in at least one tumour xenograft. Criteria for activity in the HF assay which accomplished this goal were thus: 20 or more total points (any combination of points in the i.p. and s.c. fibres), 8 or more points in s.c. fibres, or an observation of cell kill in any fibre in either the i.p. or s.c. compartment.

The NCI in vitro anti-cancer drug screen has been described in detail previously (Alley et al, 1988; Monks et al, 1991). It utilizes 60 different human tumour cell lines representing leukaemia, melanoma, lung, colon, brain, ovary, breast, prostate and kidney cancers. For a typical screening experiment, cells are inoculated into 96-well microtitre plates and allowed to incubate for 24 hours. Experimental drugs are solubilized in dimethyl sulfoxide and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted with complete medium. 4,10-fold or 1/2 log additional serial dilutions are made to provide a total of 5 drug concentrations plus control. Following drug addition, the plates are incubated for an additional 48 hours. Sulforhodamine B solution is added to each well, and bound stain is solubilized and the absorbance read. The percentage growth is calculated at each of the drug concentration levels. 3 dose response parameters are calculated for each experimental agent: growth inhibition of 50% (GI_{50}), total growth inhibition (TGI), and the concentration of drug resulting in a 50% reduction in measured protein (LC_{50}).

Structural characteristics

Chem-X, a product of Chemical Design, Ltd (Oxfordshire, UK), was used to quantitate aspects of structural characteristics such as the number of hydrogen bonds, hydrogen bond donors and acceptors. Molecular weights were taken as calculated from molecular formulas from the DTP Drug Information System.

Statistical tests

χ^2 analyses were conducted to examine the effect of various factors on in vivo outcomes; where insufficient data were available for a χ^2 analysis, the Fisher's exact test was employed. The Spearman rank correlation coefficient was used to correlate in vivo activity with clinical responses; this statistic is defined as the more familiar Pearson correlation of the data, after the data values for each measure of activity have been replaced by their respective ranks. The Spearman rank correlation is more appropriate than the Pearson correlation in situations where the data are not normally distributed. A Student's *t*-test was used in comparing mean MW and means for hydrogen-bonding characteristics. Statistical significance was set at the 99% confidence level ($P < 0.01$).

RESULTS

Indicators of clinical activity

For 39 clinical agents, relationships between xenograft response levels, averaged by histology, and the phase II response rates were investigated. The Spearman rank correlation coefficients (r) for all histologies are plotted in Figure 1. Only non-small cell lung (NSCL) xenografts were predictive of clinical activity in the same histology ($r = 0.814$, $P = 0.004$). Breast xenograft models were the most useful for predicting clinical response against any disease, correlating with clinical activity against NSCL ($r = 0.565$, $P = 0.008$), melanoma ($r = 0.540$, $P = 0.007$) and ovarian ($r = 0.611$, $P = 0.003$) cancer, but interestingly, not with clinical breast cancer. Activity in colon xenografts predicted for clinical melanoma response ($r = 0.532$, $P = 0.005$). There were no other correlations that met the criteria for statistical significance. The number of xenograft models with a T/C ≤40% or ILS ≥25% was compared with the presence of clinical activity. Clinical activity was found in only 2/6 agents (33%) with activity in fewer than one-third or more of tested xenograft models, but was found in 21/33 agents (64%) with activity in one-third or more of tested xenograft models (Figure 2A); undoubtedly due to the small number of agents, this difference is not statistically significant ($P = 0.14$). The comparison was also repeated using a more stringent definition of clinical activity, demanding that agents tested in multiple disease demonstrate response in at least 2 diseases. Applying this more strigent definition, clinical activity was found in 0/6 agents (0%) which had activity in fewer than one-third of tested pre-clinical xenograft models, whereas 15/33 agents (45%) with activity in one-third or more of tested pre-clinical xenograft models had clinical activity ($P = 0.04$) (Figure 2B). The analyses were repeated, increasing the threshold for xenograft activity to a T/C ≤10% or ILS ≥50%, a comparison which yielded no useful correlations between overall xenograft activity and either definition of clinical activity.

Indicators of xenograft activity

To assess how HF activity might be related to predicting activity in some xenograft model, we considered the likelihood of responses in xenograft models as a function of HF activity. Table 2A shows

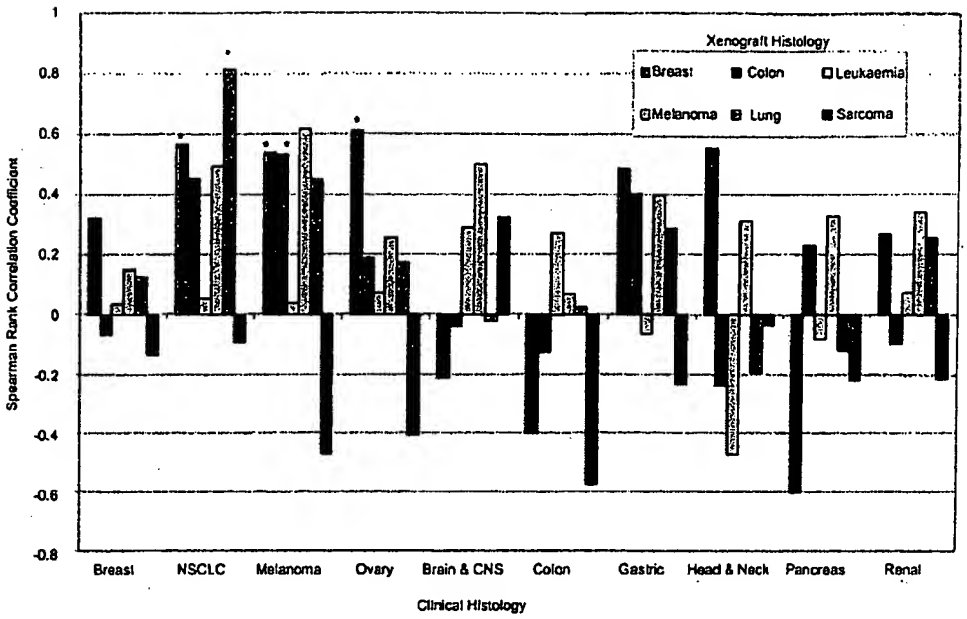

Figure 1 In vivo activity and clinical activity by disease type The median number of agents per correlation was 12 (range 2–27). For the statistically significant correlations, the median was 21 (range 10–26). The number of xenograft systems per histology ranged between 7 and 9 for all histologies except for sarcoma which included only one xenograft model. * – statistically significant correlation

Table 2A IP HF activity vs xenograft activity (at least 4 tumours tested)

	% of xenograft models active			
Responsive IP fibres	<33%	≥33%	Total	% active in 33% of xenografts
0–6 fibres	179	6	185	3%
≥7 fibers	57	14	71	20%
Total	236	20	256	8%
			$\chi^2 = 19.3$, $P < 0.0001$	

Table 2B IP HF activity vs activity in IP xenograft models

Responsive IP fibres	Agents active in IP xenograft	Agents inactive in IP xenograft	Total	% active
0–3 fibres	32	231	263	12%
4–6 fibres	23	85	108	21%
7–9 fibres	16	30	46	35%
≥10 fibres	19	23	42	45%
Total	90	369	459	20%
			$\chi^2 = 33.7$, $P < 0.0001$	

Compounds were studied in a median of one ip xenograft model, with some compounds being tested in as many as 8 models.

Table 2C IP HF activity vs activity in any xenograft model

Responsive IP fibres	Agents active in any xenograft	Agents inactive in all xenograft	Total	% active
0–3 fibres	84	212	296	28%
4–6 fibres	43	84	127	34%
7–9 fibres	26	31	57	46%
≥10 fibres	36	21	57	63%
Total	189	348	537	35%
			$\chi^2 = 28.4$, $P < 0.0001$	

that using compounds with studies in at least 4 different tumour types, there exists a strong correlation ($P < 0.0001$) between activity in HF placed in the peritoneal compartment and ultimate xenograft activity, so that compounds with activity in >6 peritoneal fibres had a 20% likelihood of subsequent xenograft activity while compounds with activity in ≤6 fibres had a 3% rate of xenograft activity. One criticism of this correlation is that it would be relevant only to intraperitoneal xenografts. Table 2B does in fact show that activity in ≥10 peritoneal fibres does, perhaps not surprisingly, show activity in 45% of peritoneal xenografts. However, Table 2C reinforces the correlation of activity in peritoneal fibres to activity in any xenograft model, including subcutaneous models. Note that for this comparison, if the threshold for HF activity is raised to more than 6 fibres, at least two-thirds of the xenograft active compounds become false negatives. Interestingly, activity in subcutaneously placed fibres did not correlate with likely activity in either peritoneal or subcutaneous xenografts (data not shown). Since HF response did not account for all of the xenograft active agents, the nature of the compounds and test conditions for these agents were examined. In all of these compounds, the xenograft activity was obtained using a route, schedule or other experimental condition not available or not routinely used in the HF assay (data not shown); in the case of some slower growing solid tumours, for example, activity was obtained using Q4Dx3 or Q7Dx3 schedules which are not available in the standard 4-day HF assay.

A second general indicator of likely xenograft activity actually emerges from consideration of in vitro screening data. Tables 3A

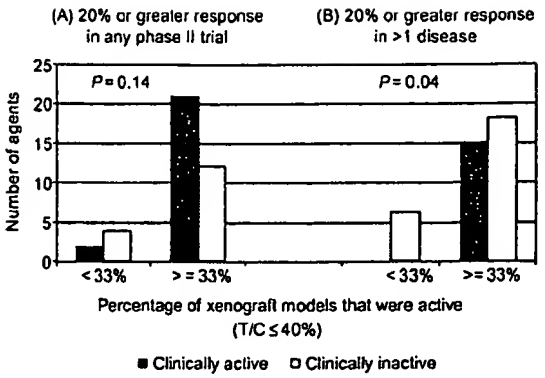

Figure 2 Overall xenograft activity & clinical activity. Agents were tested in a median of 12 xenografts (range 2–45) and a median of 4 clinical diseases (range 1–24). The 33% cutoff was chosen retrospectively to provide a useful benchmark which minimized false negatives

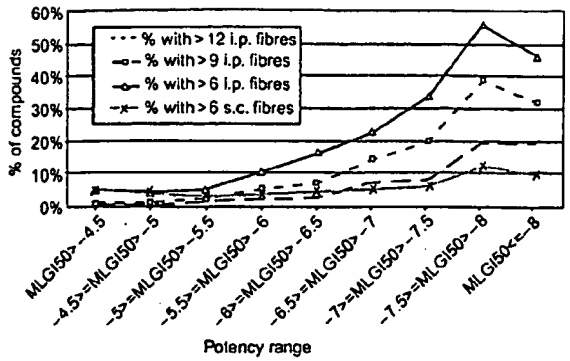

Figure 3 Mean log of GI_{50} versus percentage of compounds with hollow fiber response in at least *n* fibers

and 3B demonstrate that compounds with evidence of selective activity for successively greater numbers of lung or breast carcinoma cell lines had successively increased likelihood of demonstrating activity in lung or breast xenograft models. Compounds with evidence of in vitro selectivity in 6 or more lung cell lines exhibited activity in 33% (compared to 17.5% overall) of corresponding xenografts. Compounds with evidence of in vitro selectivity in breast cell lines were even more likely (44%) to be active in the corresponding xenografts (compared to 21% overall). When this effort was extended to other histologies, no other significant correlations emerged (data not shown).

Indicators of HF activity

Given that HF response can, to a certain extent, predict some level of xenograft activity, it becomes of interest to enquire if predictors of activity in the HF model can be gleaned from in vitro screening data or structural properties of the molecules. Table 4 demonstrates that in the case of breast, lung, ovarian, CNS and melanoma cell lines, selective activity in the particular in vitro panel does show a trend toward correlating with emergence of activity in the corresponding cell types in the HF panel. For example, 48% of the agents that were selective for breast cell line activity in vitro also showed activity (growth inhibition of 50%) in the breast carcinoma HF, compared to 39% of compounds selected for study in hollow fibres for any reason. Colon carcinoma cell line activity did not correlate with HF colon cancer cell line activity.

Greater potency in the 60 cell line assay, as indicated by the GI_{50}, correlates exceptionally well with increasing activity in the HF model with peritoneal fibres (Figure 3) ($P < 0.0001$). For example, 37% of compounds with a $\log_{10}$ GI_{50} of at least -7.5 demonstrate activity in at least 10 out of 24 i.p. fibres, whereas the fraction of all compounds with this level of activity in the HF assay is only 6%. Similar analyses were carried out utilizing activity in s.c. fibres and i.p. plus s.c. fibres. As with the comparison of HF-to-xenografts, these analyses showed that activity in the s.c. fibres did not correlate with in vitro 60 cell line potency, and that activity in the i.p. plus s.c. fibres was less correlative than the i.p. fibre response alone (data not shown).

The mean GI_{50} for only the 12 cell lines used in the HF assay was also examined to determine if this measure was more correlative than the average potency in all 60 cell lines. Although 12 cell line in vitro potency still correlates positively with HF activity, the correlation is not as striking as that obtained using the 60 cell line GI_{50} (data not shown).

'Differential' activity of a compound between distinct cell types in the in vitro screen may be an indication of a basis for expecting selective anti-proliferative effect in vivo and ultimately in the

Table 3A Analysis of in vitro lung histology response vs lung xenograft response

No. lung lines with GI_{50} < mean GI_{50}	Lung xenograft inactive	Lung xenograft active	Total tested	% active	χ^2	P
3	299	79	378	21%	9.99	0.00158
4	206	70	276	25%	24.1	<0.0001
5	133	56	189	30%	29.6	<0.0001
6	78	38	116	33%	23.8	<0.0001

Table 3B Analysis of in vitro breast histology response vs breast xenograft response

No. breast lines with GI_{50} < mean GI_{50}	Breast xenograft inactive	Breast xenograft active	Total tested	% active	χ^2	P
3	189	53	242	22%	0.665	0.415
4	159	46	205	22%	0.884	0.347
5	103	38	141	27%	5.62	0.0180
6	34	27	61	44%	25.0	<0.0001

Table 4 Histological comparison between 60 cell line screen and hollow fibre assay

HF disease type	I.	II.	χ^2	P
Breast	48%	39%	14.9	1.11E-04
Colon	26%	27%	0.182	0.670
Lung	65%	59%	10.7	0.00106
Ovarian	55%	40%	14.5	1.42E-04
CNS	58%	47%	15.9	<0.0001
Melanoma	30%	25%	8.85	0.00293

I. This column shows the activity rate for the HF tumour type where 4 or more cell lines in the same 60 cell line disease panel were responsive.
II. This column shows the overall activity rate for the HF tumour type for all compounds tested.

Table 5 Differential activity and HF activity

	Responsive IP fibres						
Δ	0	1–3	4–6	7–9	≥10	Total	% with 4 or more fibres
Δ≤.5	34	76	33	20	36	199	45%
0.5<Δ≤1	138	427	154	46	50	815	31%
1<Δ≤1.5	179	389	138	38	42	786	28%
1.5<Δ≤2	77	150	55	21	18	321	29%
Δ>2	39	97	38	6	3	183	26%
Total	467	1139	418	131	149	2304	30%
						χ^2 = 79.4, P < 0.0001	

Table 6 Molecular weight and HF activity

	Responsive IP fibres						
MW	0	1–3	4–6	7–9	≥10	Total	% with 4 or more fibres
MW≤250	47	102	27	11	2	189	21%
250<MW≤500	330	815	311	78	103	1637	30%
500<MW≤750	68	175	65	27	30	365	33%
750<MW≤1000	14	25	10	13	7	69	43%
MW>1000	8	22	4	1	7	42	29%
Total	467	1139	417	130	149	2302	30%
						χ^2 = 55.3, P < 0.0001	

Table 7 Hydrogen bonding sites and HF activity

	Responsive IP fibres						
Potential H bond sites	0	1–3	4–6	7–9	≥10	Total	% with 4 or more fibres
0 to 3	110	233	70	21	8	442	22%
4 to 6	226	577	223	53	58	1137	29%
7 to 10	105	250	97	33	59	544	35%
11 to 15	16	51	21	17	13	118	43%
≥16	10	28	7	7	11	63	40%
Total	467	1139	418	131	149	2304	30%
						χ^2 = 88.3, P < 0.0001	

Table 8 Molecular weight and HF activity (independent of hydrogen bonding sites)

	Responsive IP fibres (4–6 hydrogen bonding sites)				
MW	0	1–3	4–6	≥7	Total
MW≤250	24	41	15	4	84
250<MW≤500	178	488	186	101	953
MW>500	27	48	19	6	100
Total	229	577	220	111	1137
				χ^2 = 11.0, P = 0.0895	

Table 9 Hydrogen bonding and HF activity (independent of MW)

	Responsive IP fibres (250 <MW ≤500)					
Potential H-bond sites	0	1–3	4–6	7–9	≥ 10	Total
0–3	86	163	55	14	8	326
4–6	175	488	188	45	56	952
≥7	69	164	68	19	39	359
Total	330	815	311	78	103	1534
					χ^2 = 35.8, P < 0.0001	

clinic. Indeed, the potential for this to emerge was a key rationale for embarking on the 60 cell line screening paradigm (Alley et al, 1988). The difference in GI_{50} for any cell line and the average GI_{50} for all 60 cell lines was computed for the compounds studied in the peritoneal fibres. The largest differential or 'Δ' over the 60 cell lines is recorded by cell type and value for each compound. Table 5 shows that increasing Δ actually correlates negatively with increasing likelihood of observance of activity in the HF model (P < 0.0001). For example, the percentage of compounds which respond in at least 4 i.p. fibres is greater with a Δ of ≤0.5 (45%) than the percentage with Δ > 2 (26%). The same trend is displayed when comparing Δ with xenograft activity, but the correlation is not statistically significant (data not shown).

The effect of molecular weight and the number of potential hydrogen bonds in the compound structure on activity in the HF assay were also examined. Compounds in low MW ranges (160 to 480) are generally considered to be more 'drug-like' than those in higher ranges (Ghose et al, 1999). Interestingly, the number of responsive i.p. fibres generally increases with increasing MW up to a MW of 1000 (Table 6). For MW greater than 1000 activity appears to decrease, but there are too few compounds of this size level to generalize. In molecules with 7 to 10 potential hydrogen bonds, 35% of compounds responded in a least 6 i.p. fibres versus 30% overall (P < 0.0001) (Table 7). This percentage increased to 43% in compounds with 11 or more potential hydrogen bonds. Since, however, the number of heteroatoms contributes to both the number of hydrogen bonds and MW, interdependence of MW and hydrogen bond counts was examined and the results shown in Table 8. Using the constant hydrogen bond range of 4 to 6, the correlation between MW and HF activity was no longer statistically significant (P = 0.0895). However, using the constant MW range of 250 to 500, the hydrogen bond parameter, now independent of the effect of MW, still correlated strongly with HF response (P < 0.0001) (Table 9). Neither MW or the number of hydrogen bonds had any influence on activity in the s.c. fibres. The

relationship between these physiological properties and xenograft activity had also been examined; although the same conclusions could be drawn, the correlations were not as strong (data not shown).

According to the Lipinski Rule to Five (Lipinski et al, 1997), compounds with more than 5 hydrogen bond donors (HBD) or more than 10 hydrogen bond acceptors (HBA) are more likely to have poor oral absorption. Among the compounds tested in the HF assay, 95% had less than 5 HBD and 95% less than 10 HBA; since the percentages of compounds with favourable HBD and HBA counts was so great, an analysis of the effect of these parameters was not performed. In fact, these parameters were identical to the compounds entering the in vitro screening system.

Unfortunately, only about 75% of compounds selected for the HF assay are tested in that system, primarily due to problems with insufficient supply of compound. The 25% of selected but not tested compounds represent a potential source of bias in these analyses, particularly as the compounds for which it is not possible to obtain the quantities necessary for in vitro testing might represent greater structural complexity. In fact, the mean MW of non-tested compounds was 485 (vs 420 for tested compounds), with only approximately 1.3 additional H bond sites per molecule (data not shown).

DISCUSSION

The analysis of xenograft versus clinical results illustrates that a histology to histology comparison of these models to activity in the clinic cannot be reliably discerned for these 'empirically' selected compounds acting against non-molecularly characterized tumours. Although, with the exception of lung, histological matches were not found between in vivo models and clinical response, activity in multiple xenograft models does appear to predict for some degree of clinical activity. Interestingly, increasing the activity threshold to a T/C of ≤10 did not increase the likelihood of a positive clinical outcome, indicating that this activity threshold is probably too stringent. Requiring greater clinical activity (activity in 2 or more diseases) does improve the correlation between xenograft activity and clinical activity. Although the more stringent definition of clinical activity may not be suitable for making decisions on whether to advance agents from phase II to phase III trials, as a purely statistical exercise, it does support the conclusion that activity in multiple xenograft models is a useful predictor of clinical activity.

In contrast to results achieved with the DTP xenograft system, Fiebig (Scholz et al, 1990) has developed xenograft tumours which retain characteristics similar to the clinical specimens, having been grown from slow-growing and well-differentiated cell lines. Fiebig reports that when treating these xenograft tumours with the same standard agents as were utilized in the clinic for the corresponding patient tumours, the response of these xenografts in comparison to patient tumours was 90% (19/21) for sensitive and 97% (57/59) for non-responding tumours respectively. While this degree of correspondence between clinically used agent activity in xenografts and the clinic is gratifying, it is uncertain how to translate that experience to the evaluation of new agents with no prior defined clinical activity. As the clinically used standard agents have in all cases proven active in many xenograft models, this result at one level might be in accord with our finding that the number of xenografts in which an agent is active correlates with likely activity in the clinical setting.

The results presented in Figures 1 and 2 may be taken to argue against the use of activity in an empirically selected xenograft model to predict activity in the same histologic type of cancer in the clinic, and indeed that result has influenced the current philosophy underlying NCI's drug discovery and development programme (Sausville and Feigal, 1999). Nonetheless, definition of an active agent in xenografts would allow optimization of schedule, assessment of molecular target endpoints, and increase the probability of clinical activity. It is therefore beneficial to have the ability to select agents with a likelihood of xenograft activity using a rapid and inexpensive test. The HF assay was developed to serve as a discriminator for compounds emerging from an empirical in vitro cell line screen. Our analyses indicate that greater levels of response in the i.p. fibres correlate with greater likelihood of xenograft activity. The same cannot be stated for responses in the s.c. fibres. Double (Phillips et al, 1998) reports that the NCI protocol for HF does not allow sufficient time for angiogenesis to occur, and hypothesizes that s.c. response may be underestimated with NCI's protocol. Folkman (Hahnfeldt et al, 1999), however, claims that a tumour does not require vessels until it is 3–4 mm in diameter; the polyvinylidene fibres are only 1 mm. Subcutaneous response is dependent on extravascular drug concentration, so the lack of correlation between s.c. fibres response and xenograft activity may be speculatively related to factors such as dose, route, schedule, hydrostatic pressure and hydration state of the host.

As with xenograft to clinical comparisons, activity against a single specific HF histology was generally not useful for predicting xenograft activity in the same histology. The lack of histologic correlations in this case can perhaps be explained by the minimal number (2 of each histology) in the HF assay. In contrast, most panels in the 60 cell line in vitro assay consist of a minimum of 6 different cell types, so cell panel specificity is more readily defined. Note that the correlations between in vitro and either HF or xenografts are only apparent when 4 or more cell lines respond.

The examination of the in vitro screening data indicate strong histologic correlations with HF activity for all panels but colon, while indications of differential activity (high 'Δ'), did not correlate with HF activity. This observation combined with that of the correlation of HF response with increasing in vitro potency may indicate that the standard HF assay is sensitive to strongly cytotoxic as opposed to 'differentially' acting agents. It can also be taken to indicate that if differential activity is truly suggested from in vitro results, a HF experiment addressing that particular set of cell types may need to be designed or specific target manipulation should be considered.

Our analysis of MW and hydrogen-bonding factors revealed a correlation between increasing MW, as well as greater hydrogen-bonding sites and HF activity. This suggests that in choosing test agents for further development, a compromise should be made between low MW for drug development purposes and the number of hydrogen-bonding sites for efficacy. HBA and HBD counts were not revealing, as 95% of the input to the in vitro screen was in the favourable range for these factors.

These results may be considered to define a 'road-map' for charting the transition of a compound from an in vitro screening result to a clinical candidate. If the development strategy of a compound is to remain empiric, consideration of the heterogeneous nature of in vivo model to clinical correlation might require strategies to define the likelihood of clinical activity, and again overall potency and activity in a large number of HF fibres might allow the best delineation of compounds to consider for further

development. It could be argued from this experience that compounds with an anti-proliferative effect at 10^{-6} M have a relatively higher likelihood of affecting all growth in a responsive tumour. Compounds of the future will likely be advanced to clinical testing with an eye toward addressing a defined molecular abnormality or target in the tumour cell. Greater numbers ($n \geq 4$) of redundant cell types expressing the target, whether in an in vitro assay such as the 60 cell line screen, or an in vivo assay such as the HF assay, might be expected to have concordant effects in xenografts bearing the same target. Finally, as efforts are consolidated to derive engineered animal strains to provide models of tumour biology and pathophysiology (http://www.nci.nih.gov/dcb/odhome.htm#MOUSE), consideration of the 'baseline' experience of an empirically oriented drug discovery program might usefully benchmark the types of compounds suitable for advancement to such models.

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